# The human exosome and PNPase

## Molecular machines for controlled RNA degradation

Reinout Raijmakers

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## Molecular machines for controlled RNA degradation

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**Promotor:**

Prof. Dr. W.J. van Venrooij

**Copromotor:**

Dr. G.J.M. Pruijn

**Manuscriptcommissie:**

Prof. Dr. W.W. de Jong
Prof. Dr. H.G. Stunnenberg
Dr. B.G.M. van Engelen

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*Dr. Dilbert Doppler*: She has lost her mind!

*Jim Hawkins*: Well, *you* can help her!

*Dr. Dilbert Doppler*: Quit it Jim! I´m an astronomer, not a doctor. I mean, I´m a doctor, but not *that* kind of a doctor. I have a doctorate, its not the same thing. You can't help people with a doctorate, you just sit there and you're useless. *[Dr. Dilbert Doppler holds his hands in front of his face and starts crying.]*

*Jim Hawkins*: It's ok doc, its allright.

**Treasure Planet** - Walt Disney Pictures

De Sint is al oud en heeft in zijn leven veel gestudeerd,
maar een aantal zaken krijgt hij maar niet aangeleerd.
Humaan exosoom, een complex van exoribonucleases,
en dat vormt met bepaalde eiwitten associaties.
In de nucleolus vertonen die eiwitten accumulatie,
maar dat is weer afhankelijk van de conformatie.
Daar bij komt dat een exosoom kan coprecipiteren,
terwijl bepaalde deletiemutanten dat niet presteren.
Uit dat alles kan dan worden geconcludeerd,
dat inderdaad het exosoom en eiwit zijn geassocieerd.
...

**Sinterklaas**, December 2001

*Cover Illustrations*

*Front*

Closeup of the right eye of *Spoekie*, a common houserabbit (*Oryctolagus cuniculus*), to remind that the research in this thesis would not have been possible without the sacrifice of rabbits H69, H70, H71, H72, H73, H74, SN565, SN566, SN567 and SN568. Photography by Erik Vossenaar (2003).

*Back*

Several photographs of the rabbits *Spoekie*, *Zowie*, *Tuzi*, *Dink* and *Flynn*, all showing that working together is much more fun!

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# Chapter 1

## General Introduction

Although DNA (deoxyribonucleic acid) and proteins are probably the most generally known biomolecules (one carrying the genetic code, the other because enzymes, which are usually proteins, are nowadays included in a lot of consumer products), a third biomolecule called RNA (ribonucleic acid) is at least equally important for life on earth. It is generally assumed that RNA was the first biomolecule in the evolution of life and many types of RNA still play an important role in various cellular processes, particularly as an intermediate in the translation of the genetic code into functional proteins. For example, small nucleolar RNAs (snoRNAs) are involved in the generation of ribosomal RNA (rRNA), which are molecules that are part of the ribosome, the machinery that translates messenger RNAs (mRNAs), copies of the genetic code in the DNA, into proteins. In addition, transfer RNAs (tRNAs) bring the amino acids to the ribosome. The production of mRNA involves not only proteins and DNA, but also small nuclear RNAs (snRNAs) to obtain the mature messengers. All these processes are carefully regulated and cellular levels of these RNAs must therefore be tightly controlled. One of the most important protein complexes in human cells involved in maintaining correct RNA levels is called the exosome. This chapter summarizes our current knowledge on structure and composition of this complex and explains the role of the complex in both the production and degradation of various types of RNA molecules.

## Brief history of the human exosome

The human exosome, originally called the PM/Scl complex, was first identified as an autoantigen in 1977, when specific autoantibodies in sera of patients suffering from polymyositis (PM) and overlap syndromes precipitated an antigen ('PM-1') from calf thymus extract (1). Later it became apparent that especially sera of patients with an overlap syndrome of (poly)myositis and scleroderma (Scl) contained reactivity with this antigen, which was therefore termed the 'PM/Scl antigen' (2,3). The PM/Scl antigen was shown to be a nucleolar complex (4), consisting of 11-16 proteins ranging from 20-110 kDa (5,6). After the identification and cloning of the main autoantigenic proteins, PM/Scl-100 and PM/Scl-75 (7-9), both these proteins were shown to be homologous to bacterial exoribonucleases (RNase D and RNase PH, respectively)(10).

In recent years, the PM/Scl complex was shown to be the human equivalent of the yeast exosome, a complex consisting of a large number of exoribonucleases, involved in the degradation and processing of many different RNA species (11).

## Composition and structure of the exosome

### *Components of the exosome complex*

Originally described in the yeast *Saccharomyces cerevisiae,* the exosome complex was shown to consist of nine core components of which six are homologous to bacterial RNase PH (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p) and three contain a putative S1 RNA binding domain (RBD) (Rrp4p, Rrp40p and Csl4p)(11-13). In addition, the RNase D homologue Rrp6p was found only in the nuclear exosome complex and Rrp44p/Dis3p (homologous to bacterial

RNase R) in both nuclear and cytoplasmic exosome complexes (13). For all these proteins, human homologues have been described and with the exception of hRrp44p/hDis3p, all of these proteins were shown to be present in the human exosome (11,14-17). Four yeast exosome proteins (Rrp4p, Rrp41p, Rrp44p/Dis3p and Rrp6p) and two human exosome proteins (OIP2, the human homologue of Rrp43p, and hRrp4p) have been shown to display 3'-5' exoribonuclease activity in vitro (12,18,19). However, based on the homology between the exosome proteins, the majority of the other subunits is predicted to have a similar enzymatic activity. An overview of the proteins known to be incorporated in the human and yeast exosome is given in Table 1.

Recently, the core components of the yeast exosome were shown to assemble in a donut-shaped structure, with the six RNase PH homologous proteins forming a hexameric ring and the three S1 RNA binding proteins on top of this ring, a structure similar to that of the bacterial protein polyribonucleotide phosphorylase (Figure 1) (20,21). The nature of the association of the Rrp6p and Rrp44p/Dis3p proteins with the core of the exosome complex has not been characterized yet.

### *The exosome in other organisms*

Besides yeast and human, the exosome has been characterized in a number of other organisms. In archaebacteria, a similar complex has been suggested to exist, consisting of only three different proteins, two homologous with RNase PH and one containing an S1 RBD (22). The *Trypanosoma brucei* exosome contains six RNase PH homologous proteins, three proteins with an S1 RNA binding domain and homologues of the yeast proteins Rrp6p and Lrp1p (23,24). The exosome of *Drosophila melanogaster* contains homologues of all yeast exosome proteins except for Rrp43p and Rrp45p (25). Also in Arabidopsis thaliana homologues of Rrp4p and Rrp41p have been characterized (22,26). The fact that not all organisms contain six RNase-PH like proteins and three S1 RBD proteins, which in the (yeast) exosome assemble into such a well conserved structure, suggests that in some organisms multiple copies of specific proteins might be present in the fully assembled exosome.

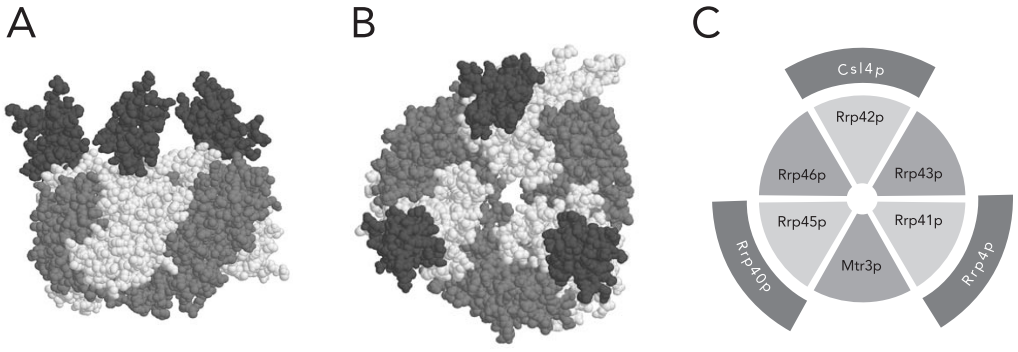


**Figure 1. Model of the yeast exosome complex.**
A three-dimensional model of the core of the yeast exosome generated based upon the structural homology between the exosome and PNPase (21). Panel A: side view; panel B: top view. Panel C shows a schematic model of the arrangement of the nine core proteins in the yeast exosome. The six RNase PH-like proteins, forming a hexameric ring, are shown in semi-white and light gray, the three proteins with an S1 RNA binding domain, associated with one side of the ring, are shown in dark gray.

**Table 1. Components of the exosome complex**

| human protein[1] | yeast equivalent[1] | conserved domains | activity (hypothetical or *experimental*) | comments |
|---|---|---|---|---|
| *core proteins* | | | | |
| hCsl4p | Csl4p/Ski4p | S1 | RNA binding | |
| hRrp4p | Rrp4p | S1 / KH | *3'-5' hydrolytic exoribonuclease* | |
| hRrp40p | Rrp40p | S1 / KH | 3'-5' hydrolytic exoribonuclease | |
| hRrp41p | Rrp41p/Ski6p | RNase PH | *3'-5' phosphorolytic exoribonuclease* | |
| hRrp42p | Rrp42p | RNase PH | 3'-5' phosphorolytic exoribonuclease | |
| hRrp46p | Rrp46p | RNase PH | 3'-5' phosphorolytic exoribonuclease | |
| hMtr3p | Mtr3p | RNase PH | 3'-5' phosphorolytic exoribonuclease | |
| OIP2 | Rrp43p | RNase PH | *3'-5' phosphorolytic exoribonuclease* | |
| PM/Scl-75 | Rrp45p | RNase PH | 3'-5' phosphorolytic exoribonuclease | binds to AU-rich RNA sequences |
| *other common proteins* | | | | |
| PM/Scl-100 | Rrp6p | RNase D | *3'-5' hydrolytic exoribonuclease* | has been suggested to be only in the nuclear exosome |
| (hDis3p) | Rrp44p/Dis3p | RNase R | *3'-5' hydrolytic exoribonuclease* | human protein not observed in complex |
| *associated proteins* | | | | |
| hSki2w | Ski2p | DEAD box | RNA helicase | Ski2p/Ski3p/Ski8p cytoplasmic complex |
| (KIAA0372) | Ski3p | TPR repeats | unknown | Ski2p/Ski3p/Ski8p cytoplasmic complex |
| (REC14) | Ski8p | WD40 repeats | unknown | Ski2p/Ski3p/Ski8p cytoplasmic complex |
| (eRFS) | Ski7p | GTPase | unknown | associated with the cytoplasmic exosome |
| MPP-6 | - | - | unknown | no yeast homologue known |
| ISG20 | (Rex4p) | ExoI/II/III | *3'-5' exonuclease* | |
| (KIAA0052) | Mtr4p/Dob1p | DEAD box | RNA helicase | associated with the nuclear exosome |
| (Ran) | Gsp1p | GTPase | *GTPase* | interacts with Dis3p |
| (CGI-37) | Nip7p | - | unknown | interacts with Dis3p |
| Rpp14 | - | - | *3'-5' phosphorolytic exoribonuclease* | interacts with OIP2 |

[1] If the name is between brackets, the association is based on homology only.

*Specificity of the exosome*

Apart from the proteins homologous to known exoribonucleases, a number of other proteins have been found to be associated with the exosome that might play a role in the specificity of the complex (Table 1). In yeast, the ATP-dependent RNA helicase Mtr4p/Dob1p has been implicated in the specificity of the nuclear exosome complex (27). The cytoplasmic yeast exosome associates with the putative GTPase Ski7p (28) and a complex of three proteins (Ski2p, Ski3p and Ski8p), one of which is also a putative RNA helicase (29). The human homologue of this RNA helicase, hSki2w, has also been found in the human exosome (17), but localizes to both the nucleolus and the cytoplasm (30).

## Functions of the exosome

The exosome has in recent years shown to be involved in a wide variety of processes. It is present in the cytoplasm, nucleoplasm and nucleolus of eukaryotic cells and functions in both biogenesis and degradation of many RNA species. Most of the functions of the exosome described below have been identified in yeast, but the same multi-functionality is also believed to apply on the human exosome.

*rRNA processing*

The first function attributed to the exosome in *S. cerevisiae* was its role in the maturation of the 5.8S ribosomal RNA (12,31). Of the four RNA molecules present in fully assembled eukaryotic ribosomes (5S, 5.8S, 18S and 28S in human), three are transcribed by RNA polymerase I as a single precursor RNA which is subsequently processed to the mature ribosomal RNAs (Figure 2). Yeast strains lacking exosome core subunits or proteins associated with the nuclear exosome (Rrp6p, Dis3p, Gsp1p or Mtr4p) tend to accumulate 5.8S rRNA molecules that are extended at the 3' end (the 12S RNA in human and the 7S RNA in yeast) (27,32-35). In the absence of these proteins, also other incomplete or incorrectly processed rRNA precursors, that normally are rapidly degraded by the exosome complex, tend to accumulate as well (23,27,34-36).

*Small RNA processing*

The processing of the pre-rRNA is also mediated by a significant number of small nucleolar RNAs (snoRNAs) and their associated proteins. These complexes function in endonucleolytic cleavage of the RNA at specific sites and the 2'O-methylation and pseudouridinylation of specific residues. In the nucleoplasm, a number of small RNAs (snRNAs) is involved in the splicing of pre-mRNA. Usually, these small RNA molecules are transcribed by RNA polymerase II or III and most of them require post-transcriptional modification (37). In many cases, like the U1, U4 and U5 snRNAs and a wide variety of snoRNAs (including the U3 snoRNA), the nuclear exosome is involved in the maturation of the 3' end of these RNA molecules (33,38,39), although also other exoribonucleases may participate in these processes (40). One of the human exosome subunits, OIP2, has been reported to interact with Rpp14, a protein associated with the RNase P and RNase MRP complexes, which are involved in the maturation of tRNA and rRNA, respectively(19,41). Finally, the exosome and especially the Rrp44p/Dis3p subunit might play a role in the biogenesis of the small RNA component of the cytoplasmic signal recognition particle (SRP), which is assembled in the nucleolus of eukaryotic cells (42,43).

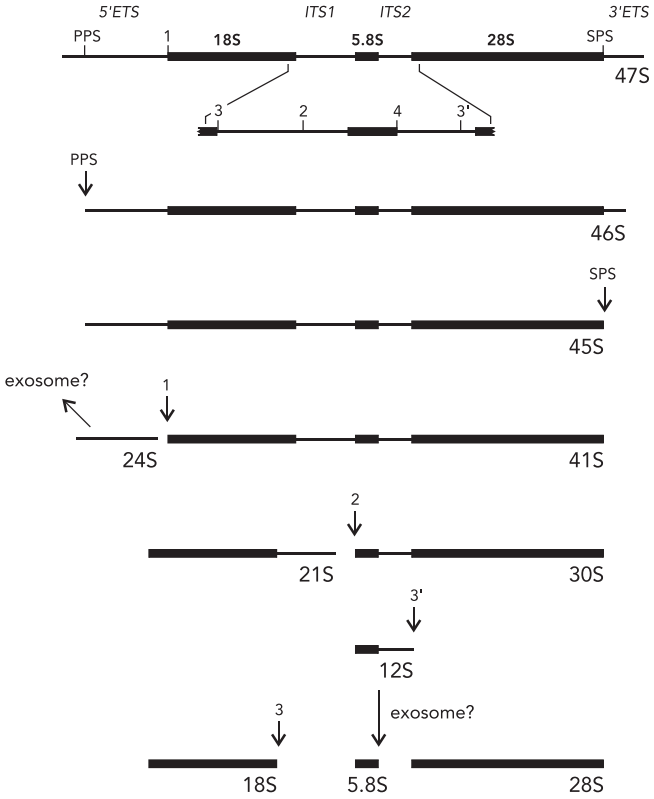


**Figure 2. Pre-rRNA processing pathway in human.**
A schematic representation of the processing of the human pre-ribosomal RNA into three mature rRNAs, 5.8S, 18S and 28S. The pre-rRNA is transcribed as a single precursor, the 47S pre-rRNA. Endonucleolytic cleavages at the primary processing site (PPS) and secondary processing site (SPS) initiate the processing. Subsequently, cleavage at sites 1 and 2 separates the 21S precursor, which matures into the 18S rRNA, from the 30S precursor. The latter is finally converted into the 28S rRNA and the 5.8S rRNA. Based on yeast experiments, the exosome complex is predicted to be involved in the final maturation step of the 5.8S rRNA and in the degradation of the 5' ETS sequence.

*Quality control of pre-mRNA*

Upon transcription of a precursor mRNA molecule by RNA polymerase II, the pre-mRNA has to be capped, spliced and generally poly-adenylated before it can be transported to the cytoplasm to be translated. Many of these maturation steps are subjected to quality control of the pre-mRNA and if a problem is encountered, the RNA will be degraded. An overview of the steps of this quality control process involving the exosome in yeast is shown in Figure 3. Immediately following transcription, pre-mRNAs with an extended 3' UTR (in cases where the RNA polymerase failed to stop at the correct position) are recognized by the nuclear exosome complex. The core of the exosome degrades the additional 3' sequence to approximately the correct end of the pre-mRNA. These RNAs can then be further processed to functional pre-mRNA molecules, or they are handed-over to the exosome subunit Rrp6p, which degrades the rest of the RNA (44). After the splicing and polyadenylation, a second checkpoint involving both Rrp6p and the core of the exosome is used to prevent hyperadenylated

mRNAs or mRNAs without any poly(A) tail from leaving the site of transcription, possibly by removing (part of) the 3' UTR of the mRNAs (18,45,46). If the poly(A) tail is correct, a complex of Rrp6p, poly(A) polymerase and the hnRNP protein Npl3p hands the mRNA over to a number of proteins (Hpr1p, Sub2p, Yra1p), capable of binding the 3' UTR of the mRNA, which are involved in the export of the mRNA to the cytoplasm (18,47). The mRNA is recruited to the cytoplasmic ribosomes to be translated for the first time, although recent evidence suggests that also a nuclear translation machinery might be involved in this quality control step (48). If the mRNA contains premature stop codons (e.g. due to incorrect splicing), it is rapidly shifted into the normal turnover pathway for mRNA, preventing further translation (a process called nonsense mediated decay, NMD) (49). If, however, the mRNA somehow lacks a normal stop codon, a different pathway is used to degrade the mRNA, called non-stop decay (50,51). In this pathway, the cytoplasmic exosome and its associated proteins degrade both the poly(A) tail and the rest of the mRNA. Messenger RNAs that can be translated correctly the first time are often translated multiple times, before also they are degraded by the normal mRNA turnover mechanism.

With regard to mRNA quality control in other organisms, a physical link between the exosome and the RNA polymerase II transcription machinery was recently shown in *D. melanogaster* (25). In addition, the human exosome subunit PM/Scl-75 has been shown to interact directly with two transcription co-factors, E12 and E37 (52).

*mRNA turnover*

The normal pathway for mRNAs to be degraded starts with the removal of the poly(A) tail, followed by decapping and degradation in either a 5'-3' or a 3'-5' direction (53,54). These processes are mediated by the exosome and possibly regulated by the cytoplasmic Lsm complex (55). Whereas in yeast the main turnover pathway starts at the 5' end of the mRNA and only a minority of the mRNAs are degraded by the exosome, in mammalian cells most of the mRNA is degraded by the 3'-5' exoribonucleases of the exosome (56,57). Following deadenylation, the mRNA is rapidly degraded by the exosome complex, finally resulting in the hydrolysis of the 5' cap structure (56,58). The recruitment of the exosome to mRNAs that have to be degraded in yeast is most likely mediated by sequence specific RNA binding proteins, possibly via Ski7p and the Ski2p/Ski3p/Ski8p complex (53). In human cells, the degradation of inherently instable mRNAs that contain AU-rich elements (AREs) requires only the exosome (containing hSki2w) and one or more AU binding proteins (17). In addition, it has been shown that the exosome subunit PM/Scl-75 is capable of binding directly to mRNAs containing AU rich sequences (57).

*Anti-viral protection*

A number of the subunits of the yeast exosome and its associated proteins were originally described as one of eight so-called SKI genes (SKI1-SKI8) in yeast. Loss of any SKI gene function gives rise to the super killer phenotype, which is characterized by an enhanced replication of dsRNA viruses in infected $ski^-$ strains compared to infected wild-type strains (59). It is now known that all of these proteins, except Ski1p, are somehow associated with the exosome complex

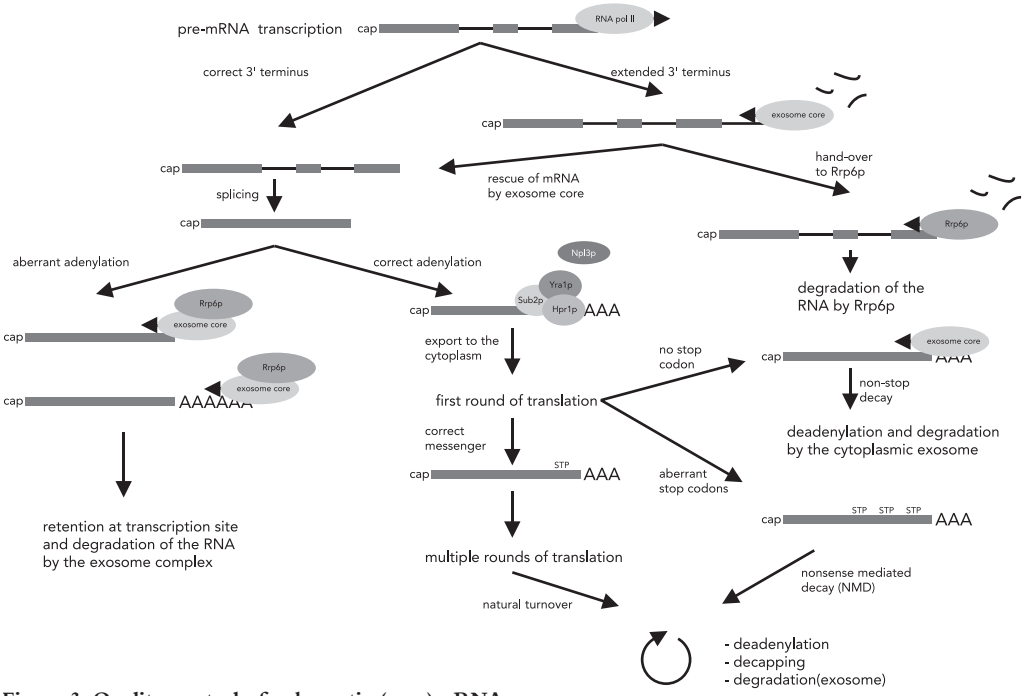


**Figure 3. Quality control of eukaryotic (pre-)mRNA.**
An overview of the steps in the quality control pathway of (pre-)mRNA in the nucleoplasm and the cytoplasm in which the exosome complex or its associated proteins are involved. The main processes involved are the degradation of mRNAs containing extended 3' termini or aberrant poly(A) tails in the nucleus, and the removal of mRNAs containing none or aberrant stopcodons in the cytoplasm by either non-stop decay or nonsense mediated decay (NMD). See text for details.

(Ski5p proved to be identical to Ski3p (60)). A model has been proposed, in which the Ski2p/Ski3p/Ski8p complex, together with Ski7p, recruits the viral RNA to the exosome complex (which contains Csl4p/Ski4p and Rrp41p/Ski6p), where it is degraded (61). In ski$^-$ mutants this degradation process is less efficient, leading to the super killer phenotype. Recently, an interferon stimulated gene (ISG20, homologous to the yeast 3'-5' exoribonuclease Rex4p), capable of protecting cells from infection with RNA viruses (62), has been found to associate with the human exosome (dr. N. Mechti, personal communication). Taken together, these data suggest that the exosome complex plays an important role in the anti-viral response in eukaryotic cells.

*Regulation of exosome function*

The variety of cellular functions in which the exosome is involved, raises the question how the complex is recruited and guided to perform a specific function. Most likely, many proteins are capable of interacting with the exosome, most of them directing a specific function or specificity for the exosome. The proteins Rrp6p and Mtr4p, for example, are only associated with the nuclear and not with the cytoplasmic complex and have very specific functions in the processing and degradation of rRNA and pre-mRNA, but not in cytoplasmic mRNA decay (13,63). The recruitment of the nuclear exosome to the site of rRNA processing might involve the protein Nip7p, which is essential for correct ribosome assembly

and has been shown to interact with the exosome subunit Rrp43p (64). As mentioned above, the Ski2p/Ski3p/Ski8p complex and Ski7p might play a similar role in recruiting the exosome to cytoplasmic mRNAs, since these proteins are required for correct mRNA degradation, but not for rRNA processing (28,53).

A special role might be reserved for the (putative RNA binding) core exosome component Csl4p, since mutants of this protein have been identified that are either defective in mRNA or rRNA processing, but not in both (53). Although many yeast proteins have been found to be associated with the exosome, only limited information is available on such proteins in human cells. The human homologue of Ski2p has been found in association with the human exosome, as well as a protein (MPP-6) of unknown function and without an obvious homologue in yeast, which is phosphorylated during mitosis (17,65). Strikingly, the yeast exosome component Rrp44p/Dis3p, of which the human homologue has not yet been linked to the exosome, is implicated to function in mitotic control and is known to interact with Gsp1p/Ran, a GTPase involved in cell cycle control (66,67). Finally, as already mentioned above, recent evidence suggests that the exosome might also be regulated by interferons, since a known interferon stimulated gene (ISG20, homologous to the yeast exoribonuclease Rex4p) has been shown to associate with the exosome complex (dr. N. Mechti, personal communication).

## Autoantigenicity of the exosome

### *Antigenicity of the complex*

Autoantibodies reactive with the human exosome are predominantly found in patients with the overlap syndrome of polymyositis and scleroderma (PM/Scl). The percentage of PM/Scl patients reported to contain antibodies capable of precipitating the PM/Scl antigen from calf thymus extract varies considerably, depending on the cohort of patients studied (12% (2), 24% (68), 76% (69)). Of the patients suffering from either scleroderma or myositis alone, only 2% and 5-9%, respectively, are reactive with the PM/Scl complex in immunodiffusion or immunoprecipitation experiments (2,68,70,71). The presence of anti-PM/Scl antibodies in the serum of patients correlates in 43-88% of the cases with the diagnosis of the polymyositis/scleroderma overlap syndrome (68,72,73).

### *Antigenicity of exosome subunits*

After the identification of the PM/Scl antigen as a multi-protein complex (5,6), two of the proteins in the complex, termed PM/Scl-100 and PM/Scl-75, were shown to contain the main autoantigenic epitopes. Anti-PM/Scl-100 and anti-PM/Scl-75 antibodies are found in 98% and 63% of the sera reactive with the PM/Scl antigen, respectively (74). The cloning of the cDNAs encoding these proteins (7-9) and the use of recombinant proteins to detect these antibodies allowed and initiated further characterization of the B-cell epitopes recognized. The main epitope on the PM/Scl-100 protein was shown to be contained in a stretch of 21 amino acids (226-246) and is recognized by 95% of all PM/Scl-100 reactive sera (75,76). Using ELISA based assays, anti-PM/Scl-100 antibodies were detected in approximately 6% of all patients with myositis, whereas anti-PM/Scl-75 reactivity was exclusively detected in sera also reactive with PM/Scl-100 (77). Most of the other human exosome subunits identified in recent years (reviewed in (78)) were shown to be autoantigenic, although

only anti-hRrp4p and anti-hRrp42p antibodies were found in a significant number of the anti-PM/Scl-100 positive myositis patients (52% and 23%, respectively) (79). Finally, anti-hRrp46p antibodies have been reported to occur in several patients with lung or prostate cancer and malignant melanoma (80).

## Outline of this thesis

### *Original goals*

When the project started in 1999, the main goals of the project were to (1) identify components of the exosome, (2) to find protein-protein interactions between exosome proteins, (3) to study how and where the exosome complex is assembled and (4) to study the function of individual exosome subunits. The first goal was met in 2001, when all the proteins present in the human exosome where published in a collaborative effort of a number of laboratories, including ours (81). Knowing all the components of the complex, a mammalian 2-hybrid system was used to identify protein-protein interactions between these protein, the second goal of the project. In Chapter 2 the interaction of one of the exosome subunits, hCsl4p, with two other subunits is described, as well as the role of different domains of the hCsl4p protein in these interactions. The elucidation of a large number of other interactions between subunits in Chapter 3 allowed the construction of a structural model for the exosome, which is also based on the homology between the exosome and a bacterial protein called polyribonucleotide phosphorylase (PNPase). In addition, the human homologue of this PNPase is described. In Chapter 5 a partially new sequence for one of the exosome subunits, PM/Scl-75, is described and compared with the originally published sequence. In contrast to the original form, this longer form of PM/Scl-75 associates readily with the exosome complex. The third and the fourth of the original goals are not extensively addressed in this thesis, although both of them are discussed in the final Chapter 8.

### *Additional goals*

During the course of any scientific project, one can encounter findings leading to new questions and consequently to new research goals. In our case, two new perspectives could be added to the project, (1) the characterization of human PNPase, a protein related to the exosome and (2) the role of the longer form of the exosome subunit PM/Scl-75 in the autoantigenicity of the human exosome. The first of these goals started with the unexpected discovery of a human equivalent of the bacterial protein PNPase. This latter protein has a structure closely related to that of the exosome complex (Chapter 3). The functional and evolutionary relationship between PNPase and the exosome is addressed in Chapter 4. The second additional goal was to determine the role of PM/Scl-75 in the autoantigenicity of the complex. In Chapter 6 the protein is shown to be cleaved during apoptosis, a process believed to play a role in the generation of autoimmunity. Finally, in Chapter 7 the new PM/Scl-75 polypeptide, which was identified in Chapter 5, is shown to be a much more important autoantigen in patients with the PM/Scl overlap syndrome, than the originally defined polypeptide. All results obtained with regard to these new goals are discussed in relation to the structure and function of the exosome complex in Chapter 8.

# Chapter 2

## Protein-protein interactions of hCsl4p with other human exosome subunits

Reinout Raijmakers
Yvet E. Noordman
Walther J. van Venrooij
Ger J.M. Pruijn

*Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, Nijmegen, The Netherlands*

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The exosome is a complex of 3′ → 5′ exoribonucleases, which functions in a variety of cellular processes, all requiring the processing or degradation of RNA. We demonstrate that the two human proteins hCsl4p and hRrp42p, which have been identified based on their sequence homology with *Saccharomyces cerevisiae* proteins, are associated with the human exosome. By mammalian two-hybrid and GST pull-down assays we show that the hCsl4p protein interacts directly with two other exosome proteins, hRrp42p and hRrp46p. Mutants of hCsl4p that fail to interact with either hRrp42p or hRrp46p are also not able to associate with exosome complexes *in vivo*. These results indicate that the association of hCsl4p with the exosome is mediated by protein-protein interactions with hRrp42p and hRrp46p.

## Introduction

The exosome was originally described as a complex of exoribonucleases in the yeast *S. cerevisiae* involved in the maturation of the 3′ end of 5.8S ribosomal RNA (rRNA) (12,31,34) (32). Additional functions for the exosome were found in the processing of small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) (33,38) and the SRP RNA (scR1) (42), the degradation of aberrant rRNA and its precursors (36) and the 3′→ 5′ turnover of (pre-) messenger RNA (mRNA) in the cytoplasm (82) and the nucleus (83). The yeast exosome contains at least 11 proteins, which all have been shown or predicted to be 3′→ 5′ exoribonucleases (11,12). In addition, several proteins have been identified that are believed to interact transiently with the exosome. A complex of Ski2p, Ski3p and Ski8p probably regulates the cytoplasmic pool of the exosome (29), whereas the nuclear exosome is controlled by Dob1p/Mtr4p (27). Some of the exoribonucleases associated with the exosome seem to have specificity for certain substrates and/or to be localized in different subcellular compartments. In yeast, the Rrp6p protein is exclusively present in the nuclear exosome complex (11) and certain mutations in the CSL4 gene can affect the turnover of mRNA in the cytoplasm, but not the nuclear processing of rRNA and snRNA (53).

By sequence homology, 11 human proteins have been identified as (putative) components of a similar complex in human cells (11,14,17). Among these homologues are the human proteins PM/Scl-100 and PM/Scl-75, which are well-known autoantigens in patients with the polymyositis/scleroderma overlap syndrome (7-9,32). It has been shown that antibodies reactive with the PM/Scl autoantigens are able to precipitate a complex of 11-16 proteins, ranging from 20-110 kDa, designated the PM/Scl complex (5,6). Recent evidence confirmed that the PM/Scl complex is in fact the human exosome (11,78). The cDNAs encoding 10 protein components have been cloned and five of them (hRrp4p, hRrp40p, hRrp41p, hRrp46p and PM/Scl-100) have been shown to be components of the human exosome (14). Functional conservation from yeast to human has been shown for four exosome proteins (hRrp4p, hRrp41p, hRrp44p/hDis3p and hCsl4p) (12,14,16,84).

No data are available yet on the architecture and structure of the exosome, neither in yeast

nor in higher eukaryotes. Here we demonstrate that hCsl4p directly interacts with both hRrp42p (which is identical to KIAA0116 (11)) and hRrp46p and that abrogation of either of these interactions prevents incorporation of hCsl4p in the exosome and also its accumulation in the nucleolus.

## Results

### *hCsl4p interacts with hRrp42p and hRrp46p*

To analyze protein-protein interactions in the human exosome, the full-length cDNAs encoding 10 (putative) human exosome components (hRrp4p, hRrp40p, hRrp41p, hRrp42p, hRrp46p, PM/Scl-75, PM/Scl-100, hCsl4p, hRrp44p/hDis3p and OIP2) were cloned into both the pACT and pBIND vectors of the Checkmate Mammalian Two-Hybrid system by fusing their open reading frames to the VP16 transcription activation domain and the GAL4 DNA binding domain, respectively. COS-1 cells were co-transfected with pairs of these cDNA constructs, followed by determination of the potentially induced luciferase activity 40-48 hours after transfection. These analyses revealed that hCsl4p interacted with two other human exosome proteins, hRrp42p and hRrp46p. Importantly, these interactions were observed both when hCsl4p was fused to the GAL4 DNA binding domain and hRrp42p and hRrp46p to the VP16 activation domain and vice versa (Fig. 1). The expression levels of the fusion proteins were monitored by western blot analysis using antibodies specific for the VP16 or GAL4 domains, which showed that all proteins were expressed at comparable levels (data not shown). To check the validity of these interactions, GST pull-down assays were performed using recombinant GST-hCsl4p fusion protein and *in vitro* translated hRrp42p and hRrp46p. As is shown in Fig. 2B, precipitation of GST-hCsl4p by glutathione-Sepharose beads resulted in efficient co-precipitation of hRrp46p and, albeit less effectively, hRrp42p, whereas in control experiments neither of these proteins was detectably precipitated by beads containing GST alone. In addition, other *in vitro* translated human exosome components were tested in this assay, but none of these proteins were specifically co-precipitated by GST-hCsl4p.

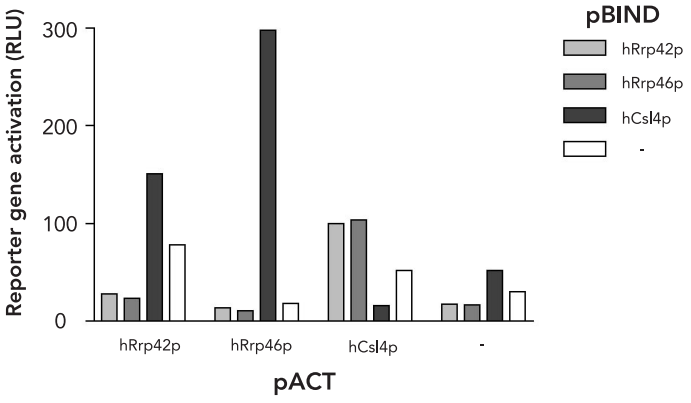


**Figure 1. Two-hybrid interactions between hCsl4p, hRrp42p and hRrp46p.**
COS-1 cells were cotransfected with constructs encoding fusion proteins of hCsl4p, hRrp42p and hRrp46p and either the VP16 transcription activation domain (pACT constructs) or the GAL4 DNA binding domain (pBIND constructs), simultaneously with a reporter plasmid. The resulting luciferase activity is depicted in relative luminescence units (RLU). The activity observed for the combination of hRrp42p in pBIND and hCsl4p in pACT was defined as 100 RLU.

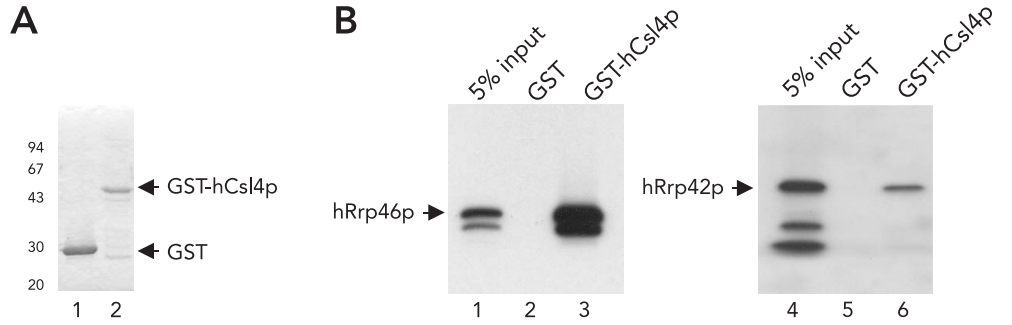


**Figure 2. GST pull-down analysis of interactions of hCsl4p with hRrp42p and hRrp46p.**
A. A fusion protein of hCsl4p and GST was expressed and purified by glutathione affinity chromatography. Approx. 1 µg purified fusion protein (GST-hCsl4p, lane 2) and GST alone as a control (lane 1) were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. On the left the positions of molecular weight markers are indicated.
B. The GST-hCsl4p and GST proteins were incubated with $^{35}$S-labeled, *in vitro* translated hRrp42p (lane 1) or hRrp46p (lane 4). After precipitation with glutathione-Sepharose beads co-precipitated proteins were analyzed by SDS-PAGE and autoradiography. Lanes 3 and 6 show hRrp46p and hRrp42p, respectively, co-precipitated with GST-hCsl4p. Lanes 2 and 5 show the corresponding controls for GST alone. The faster migrating bands observed in the *in vitro* translates are most likely caused by alternative translation initiation. In lanes 1 and 4 5% of the amount of labeled protein used for co-precipitation was loaded.

### *hCsl4p, hRrp42p and hRrp46p are associated with the human exosome*

The yeast proteins Csl4p, Rrp42p and Rrp46p all have been shown to be part of the stable 'core' complex of the yeast exosome (11,13), whereas exosome association of the corresponding human proteins has only been demonstrated for hRrp46p (14).

To investigate whether hCsl4p and hRrp42p are also part of the human exosome, which would be consistent with the observed protein-protein interactions described above, we generated transfection constructs encoding fusion proteins of each of these proteins with the enhanced green fluorescent protein (EGFP).

After expression of these fusion proteins in transiently transfected HEp-2 cells, lysates were prepared and used for immunoprecipitation experiments with a rabbit anti-EGFP antiserum. The immunoprecipitated material was analyzed by western blotting using a serum (Ven96) from an autoimmune patient strongly reactive with

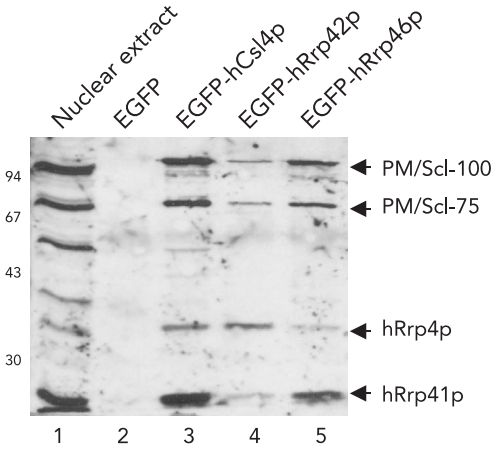


**Figure 3. Association of hRr42p, hRrp46p and hCsl4p with the human exosome.**
Co-immunoprecipitations were performed using anti-EGFP antibodies and extracts of transfected HEp-2 cells expressing either EGFP alone (lane 2) or hCsl4p, hRrp42p and hRrp46p fused to the C-terminus of EGFP (lanes 3-5). In lane 1 a nuclear extract from non-transfected HEp-2 cells was loaded. The immunoprecipitates were analyzed by western blotting using a PM/Scl positive patient serum (Ven96). Arrows indicate the positions of PM/Scl-100 and other co-precipitated autoantigenic exosomal proteins. The positions of molecular weight markers are indicated on the left.

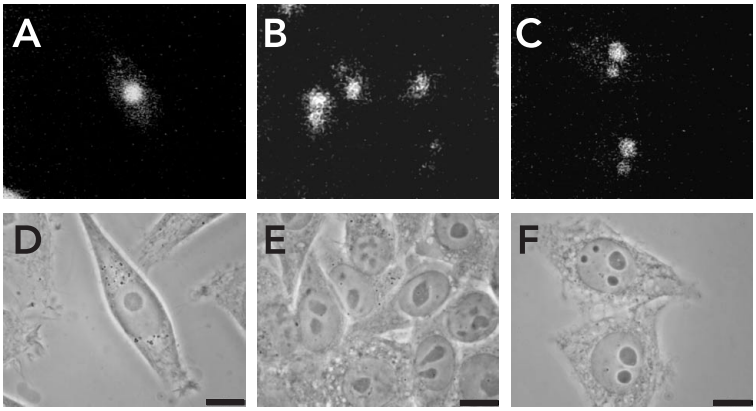


**Figure 4. Nucleolar accumulation of hCsl4p, hRrp42p and hRrp46p.**
HEp-2 cells were transfected with constructs encoding hCsl4p (panel A), hRrp42p (panel B) or hRrp46p (panel C) fused to the C-terminus of EGFP. After overnight culturing cells were fixed and the expressed fusion proteins were visualized by fluorescence microscopy. The corresponding phase-contrast images are shown in panels D-F. Each bar represents 10 μm.

the PM/Scl-100 protein and to different extends with other exosome components (including PM/Scl-75, hRrp4p, hRrp40p, hRrp41p, hRrp42p, hRrp46p, and hCsl4p). The results show that the PM/Scl-100 protein was co-precipitated with all three fusion proteins, but not with EGFP alone, indicating that in addition to hRrp46p also hCsl4p and hRrp42p are associated with the human exosome (Fig. 3). The serum reacted also strongly with 3 other specifically precipitated autoantigenic proteins. Since these proteins co-precipitate and are recognized by this serum, they are most likely other exosome components. Based on their molecular weight (approximately 28, 32 and 75 kDa) and the specificity of the serum used these proteins presumably represent hRrp41p, hRrp4p and PM/Scl-75, respectively.

The analysis of the transiently transfected HEp-2 cells expressing these EGFP-fusion proteins by fluorescence microscopy revealed a nucleolar localization for all three proteins (Fig. 4), consistent with similar analyses of other exosome components including the endogenous hRrp46p protein (14).

*hRrp46p interacts with the N- and C-terminal regions of hCsl4p*

Very little is known about the structural elements of the different exosome proteins. The hCsl4p (195 aa, 21 kDa) protein contains a region that is homologous to the *E. coli* S1 RNA binding domain (amino acids 68-147) indicating that this protein may contain RNA-binding activity. The presence of domains that could be involved in protein-protein interactions is not evident in the primary structure. To identify the regions of hCsl4p that mediate the binding to hRrp42p and hRrp46p, a set of deletion mutants was constructed as shown in Fig 5A. These mutants were created using the restriction sites BalI (hCsl4p ΔC130), PvuII (hCsl4p ΔC168), EcoRI (hCsl4p ΔC182), SmaI (hCsl4p ΔN22) and HinfI (hCsl4p Δ61-159) and were subsequently cloned into the pBind vector of the mammalian two-hybrid system, fusing them to the C-terminus of the GAL4 binding domain. The capacity of all hCsl4p mutants to interact with either hRrp42p or hRrp46p was determined by monitoring the expression of the two-hybrid reporter protein

luciferase. As is shown in Fig. 5B, deletion of either the N-terminal 22 or the C-terminal 14 amino acids of hCsl4p strongly reduced or abolished its binding to both exosome proteins. In contrast, a large deletion of the central part of the protein, comprising the putative S1 RNA binding domain, did not inhibit the interaction of hCsl4p with hRrp46p (in fact, this deletion seemed to increase the affinity for hRrp46), whereas the binding to hRrp42p was markedly reduced. Equivalent results were obtained in the reverse experiment, in which the hCsl4p mutants were cloned in the pAct vector and hRrp42p and hRrp46p in the pBind vector (data not shown).

### *Deletion mutants of hCsl4p do not associate with the exosome*

To investigate whether the amino acids deleted in the mutants of hCsl4p not only affected its interaction with hRrp42p and hRrp46p, but also its association with (functional) exosome complexes, the wild-type and mutant hCsl4p encoding sequences were cloned in transfection vectors in frame with the EGFP protein encoding sequence. After transient transfection of HEp-2 cells with these constructs, the expression of the EGFP-(mutant)hCsl4p fusion proteins was analyzed by fluorescence microscopy and western blotting. In contrast to the wild-type EGFP-hCsl4p, which accumulated in the nucleolus, all mutants showed cytoplasmic and nucleoplasmic staining with no or only weak staining of the nucleoli (Fig. 6 and results not shown). Western blotting analysis using anti-EGFP antibodies revealed that the wild-type and mutant hCsl4p proteins (fused to EGFP) were expressed to similar levels in these transfected cells (Fig. 7A). To analyze their association with exosome complexes, co-im-

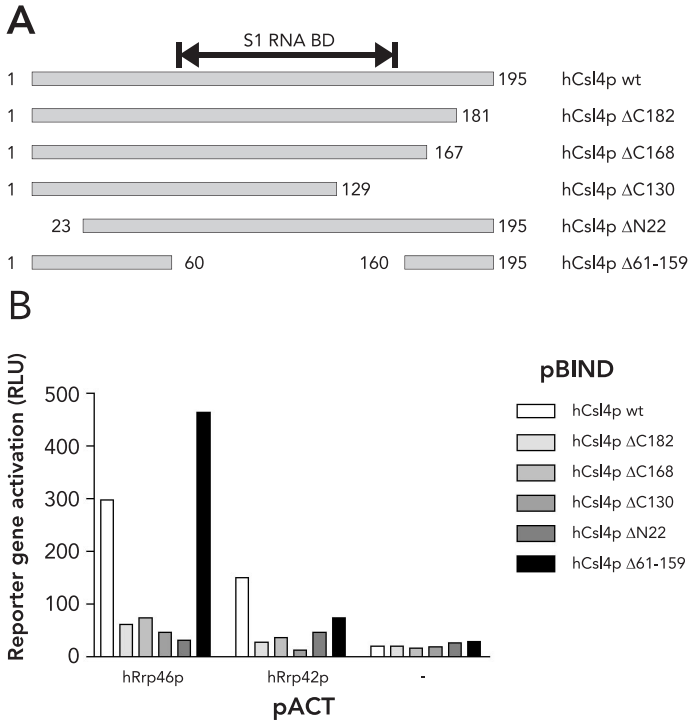


**Figure 5. Interactions of hCsl4p mutants with hRrp42p and hRrp46p.**

A. Schematic overview of the deletion mutants of hCsl4p. The position of the putative S1 RNA binding domain is indicated above the wild-type hCsl4p.

B. Two-hybrid interactions between hCsl4p mutants fused to the GAL4 DNA binding domain (pBIND constructs) and hRr46p and hRrp42p fused to the VP16 transcription activation domain (pACT constructs). The 'empty' pACT vector was included as a negative control. The luciferase activity resulting from the co-transfected reporter plasmid is depicted in relative luminescence units (RLU). The activity observed for the interaction between GAL4-hRrp42p and VP16-hCsl4p (not shown) was defined as 100 RLU.

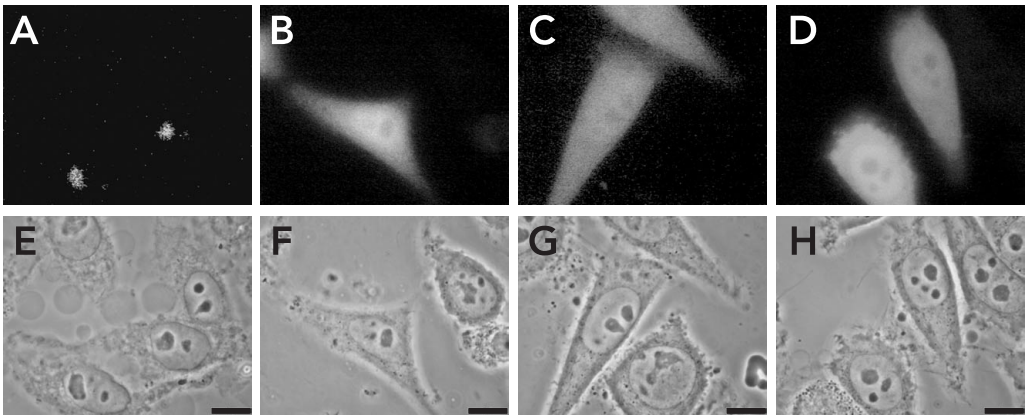


**Figure 6. Subcellular localization of hCsl4p mutants.**
HEp-2 cells were transfected with constructs encoding hCsl4p wt (panel A), hCsl4p ΔC168 (panel B), hCsl4p ΔN22 (panel C) or hCsl4p Δ61-159 (panel D) fused to the C-terminus of EGFP and the expression was visualized by fluorescence microscopy. The corresponding phase-contrast images are shown in panels E-H.

munoprecipitation experiments were performed using extracts from these cells and an anti-EGFP antiserum. Co-immunoprecipitation of exosome components was monitored by western blotting with a serum (Ven96) from an autoimmune patient, known to be reactive with several human exosome components (see above). These experiments (Fig. 7B) revealed that all hCsl4p mutants analyzed did not detectably associate with other autoantigenic exosome proteins, including PM/

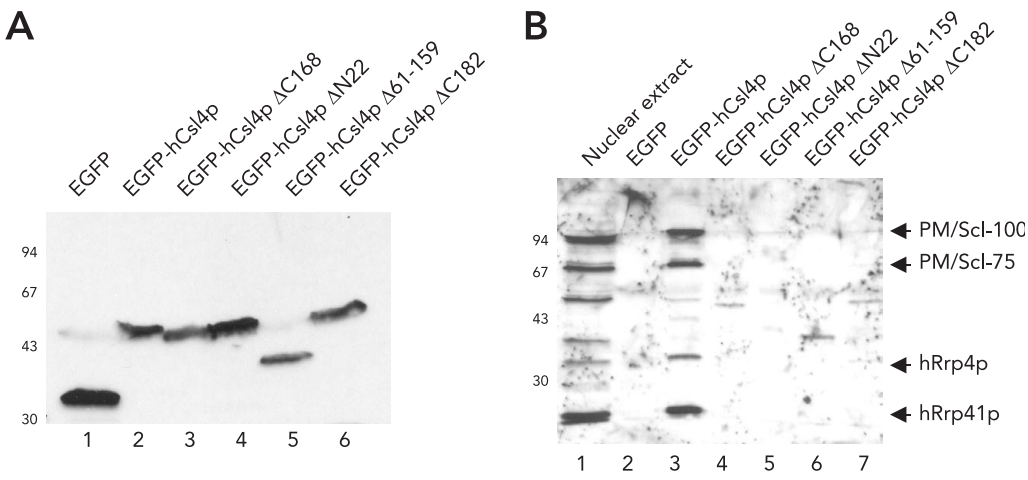


**Figure 7. Mutants of hCsl4p do not co-precipitate autoantigenic exosome components.**
A. Total extracts of HEp-2 cells transfected with constructs encoding hCsl4p mutants fused to EGFP were analyzed by western blotting using an anti-EGFP antiserum.
B. Immunoprecipitates of EGFP-tagged hCsl4p mutants from transfected cell extracts were analyzed by western blotting using a patient serum reactive with multiple exosome components. Lane 1 contains nuclear extract from non-transfected cells. Arrows indicate proteins that are co-precipitated with the wild-type hCsl4p fusion protein (lane 3) but not by any of the mutants (lanes 4-7) nor by EGFP alone (lane 2). On the left the positions of molecular weight markers are indicated.

Scl-100. The weak bands visible in the lanes 4-7 of Fig. 7B correspond to the expressed mutants of hCsl4p, that are recognized by the patient serum.

Previously it has been shown that rabbit antibodies raised against hRrp40p and hRrp46p were able to immunopurify exoribonuclease activity, most likely associated with exosome complexes (14). To investigate whether the EGFP-hCsl4p fusion protein also contained or associated with exoribonuclease activity an extract of transfected HEp-2 cells was subjected to immunoprecipitation by anti-EGFP antibodies and the immunoprecipitated material was assayed for exonuclease activity *in vitro*. The results in Fig. 8 demonstrate that the fusion protein either acts as an exonuclease or associates with other proteins displaying this activity (lanes 1-7). It should be noted that material precipitated from cells expressing EGFP alone did not display this activity, suggesting that this is due to the hCsl4p moiety of the fusion protein. When the same analysis was performed with material from cells expressing the mutant hCsl4p fusion proteins no or hardly any exonuclease activity was observed, indicating that either the association of hCsl4p with the exosome is a prerequisite for the co-precipitation of exonuclease activity or that the exonuclease activity of hCsl4p is abolished by all mutations analyzed.

## Discussion

In this paper we have identified direct interactions between three components of the human exosome, hCsl4p, hRrp42p and hRrp46p, by means of mammalian two-hybrid and GST pull-down assays. hCsl4p appeared to interact with both hRrp42p and hRrp46p, whereas no interactions between hRrp42p and hRrp46p could be detected. Deletions of different parts of hCsl4p were found to affect not only its association with hRrp42p and hRrp46p, but also

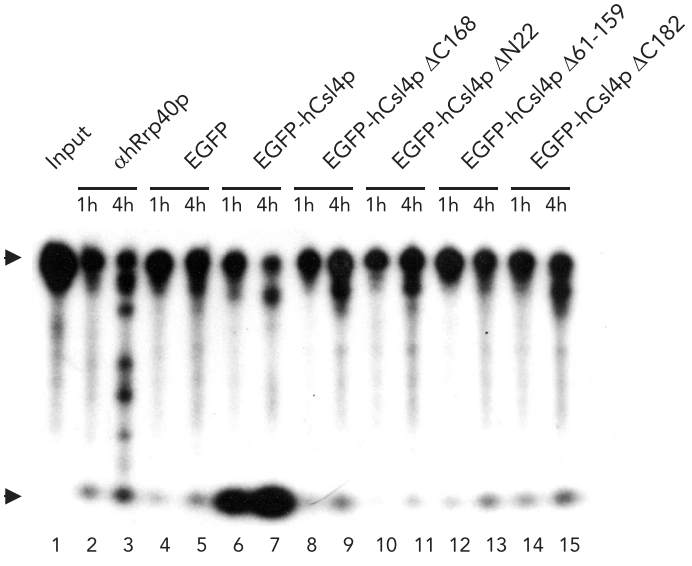


**Figure 8. hCsl4p mutants are not associated with ribonuclease activity in HEp-2 cell extracts.** Extracts were prepared from transfected HEp-2 cells expressing EGFP or the EGFP-(mutant)hCsl4p fusion proteins. Material (co-)precipitated with anti-EGFP antibodies from these extracts was analyzed for ribonuclease activity by incubating the precipitates at 37 °C with a uniformly $^{32}$P-labeled substrate RNA. Samples were taken after 1 hour and 4 hours of incubation and analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. The untreated RNA substrate (input) was loaded in lane 1 and as a positive control an anti-hRrp40p immunoprecipitate from a HeLa S100 extract was included (lanes 2 and 3). Arrows mark the positions of the substrate RNA and its major degradation product obtained after incubation with material co-precipitating with wild-type hCsl4p.

its association with exosome complexes and exonuclease activity.

### *The association of hCsl4p, hRrp42p and hRrp46p with the human exosome*

Consistent with previous observations for human exosome components (hRrp40p, hRrp41p, hRrp46p, PM/Scl-75 and PM/Scl-100) (4,7-9,14), hCsl4p, hRrp42p and hRrp46p show a mainly nucleolar localization in fluorescence microscopy. For hRrp46p it has been shown before that, despite the apparent nucleolar accumulation, the protein is also present in the cytoplasm and nucleoplasm of human cells (14). Since we provide evidence for direct protein-protein interactions between hRrp46p and hCsl4p on the one hand and hCsl4p and hRrp42p on the other, the localization of the latter two proteins may also not be restricted to the nucleolus. Moreover, the *S. cerevisiae* homologues of these three proteins are all part of the exosome complex and are located in both the cytoplasm and the nucleus of yeast cells (11). Also in *Trypanosoma brucei* both cytosolic and nuclear forms of the exosome have been reported recently, although only for hCsl4p (and not for hRrp42p and hRrp46p) a *T. brucei* homologue has been identified (23). The inability to detect hCsl4p in the cytoplasm and nucleoplasm by fluorescence microscopy is most likely due to the local concentration of hCsl4p, which is significantly higher in the nucleolus compared to other cellular compartments.

Using yeast two-hybrid systems several extensive protein-protein interaction screenings, in which all *S. cerevisiae* proteins were assayed, have been performed (67,85). In these studies a number of interactions between *S. cerevisiae* exosome proteins have been identified, which however do not include interactions of Csl4p with other proteins. Also no protein partners were found for Rrp46p, whereas in both studies Rrp42p was found to interact with Mtr3p, another protein subunit of the yeast exosome. It should, however, be noted that the functional conservation of the exosome is not necessarily associated with a conservation of the composition of this complex. This is substantiated by recent data obtained for the exosome complexes from *Caenorhabditis elegans*, *T. brucei* and archaea(23,63,86). For the *Arabidopsis thaliana* exosome subunit AtRrp41p interactions with the yeast exosome components Rrp4p and Rrp44p have recently been reported (22).

### *Several regions of hCsl4p are required for its association with the exosome and exonuclease activity*

The human hCsl4p protein is a relatively small protein of only 21 kDa, which contains a putative RNA binding domain (11,84) and at least two binding sites for other exosome proteins, hRrp42p and hRrp46p. The formation of a functional binding site for hRrp46p requires both the N-terminal and the C-terminal region of the hCsl4p protein and this interaction is apparently not disrupted by the fusion of a reporter protein (EGFP) to its N-terminus. The binding site for hRrp42p seems to be more critically dependent on the overall conformation of hCsl4p, because the binding is disrupted by non-overlapping N-terminal, C-terminal as well as internal deletions. These data also indicate that the binding sites for hRrp42p and hRrp46p on hCsl4p are at least partially distinct and that both interactions may occur simultaneously during the assembly of the exosome. Therefore it is tempting to speculate that the interactions with both hRrp42p and hRrp46p are required for hCsl4p to be incorporated into exosome complexes. The presence

of only the binding site for hRrp46p (hCsl4p Δ61-159) did not appear to be sufficient for the association with other exosome proteins.

Wild-type hCsl4p efficiently co-precipitated exoribonuclease activity. This activity appeared to be mainly processive, since the degradation products contain very few RNA fragments of intermediate size indicative of a distributive mode of action. In contrast, exosome proteins hRrp40p and hRrp46p have been shown to co-precipitate a mixture of distributive and processive exoribonuclease activities from HeLa extracts (14). A processive exonuclease remains on the RNA substrate until it is degraded to its minimal length, whereas distributive exonuclease activity involves the repetitive binding and dissociation of the protein, removing one nucleotide at a time, leading to a simultaneous degradation of the entire substrate pool. The results in Figure 8 illustrate the different patterns of degradation intermediates obtained by the hRrp40p and hCsl4p associated activities. These differences might indicate that either hCsl4p itself has (processive) exonuclease activity, or that at least two different exosome complexes exist, one enriched in hRrp40p and another enriched in hCsl4p, that display (partially) different activities. Most likely, the observed exoribonuclease activity associated with hCsl4p can not be ascribed to the hCsl4p protein itself, but instead is a function of one of the associated exosome proteins. This assumption is based on the fact that hCsl4p (as well as its yeast counterpart) does not share any homology with known exoribonucleases and that the *S. cerevisiae* Csl4p protein is the only exosome component for which no exonuclease activity has been demonstrated (13). Moreover, the recombinant GST-hCsl4p protein used in this study was unable to degrade the RNA substrate even when high amounts of protein were used (data not shown). Nevertheless, we can not rule out the possibility that lack of enzymatic activity of the recombinant bacterially expressed protein is due to the absence of essential post-translational modifications. It is tempting to speculate that the S1 RNA binding domain of hCsl4p is involved in the recruitment of substrate RNAs to the exosome, whereas the remaining parts of this protein are required for the binding of other exosome components, i.e. hRrp42p and hRrp46p, which do contain exoribonuclease activity and play a role in the degradation of substrate RNA. A similar function has been suggested for yeast Csl4p, for which it has been shown that a point mutation in its RNA binding domain does not affect rRNA processing by the exosome, in contrast to mRNA degradation. This may indicate that (h)Csl4p is somehow mediating substrate specificity of (part of) the exosome. Importantly, a mutation in the promotor region of yeast Csl4p does lead to defects in rRNA processing, indicating that the protein is required for correct assembly and/or subcellular transport of the exosome (53,84). This might be in accordance with our observation that mutants of hCsl4p lacking the binding sites for hRrp42p and hRrp46p are no longer associated with the human exosome.

These results provide the first information on direct interactions between components of the human exosome. When more of such interactions are identified, this will lead to more insight into the way the exosome is assembled, which is important for our understanding of the mechanism by which the exosome operates in the RNA degrading machinery.

## Materials and Methods

### *cDNA cloning and construction of deletion mutants*

All cDNAs used were cloned into the pAct and pBind vectors (Promega) by PCR or the use of available restriction sites. The database accession numbers for the cDNAs of hCsl4p, hRrp42p and hRrp46p are AF151866, D29958 and AF281134, respectively. Deletion mutants of hCsl4p were constructed by using suitable internal restriction sites.

### *Western blot analysis*

For western blot analysis, autoimmune patient and rabbit antisera were diluted 5000- and 500-fold, respectively, in blocking buffer (4% skimmed milk, phosphate-buffered saline (PBS), 0.1% NP-40). As secondary antibody, horseradish peroxidase-conjugated rabbit-anti human IgG or swine anti-rabbit IgG (Dako Immunoglobulins) were used after 5000-fold dilution in blocking buffer. Visualization was performed by chemiluminescence.

### *Transient transfection of HEp-2 cells and direct immunofluoresence*

For transfection, hCsl4p, hRrp42p and hRrp46p cDNAs were cloned into suitable pEGFP vectors (Clontech), allowing expression of the proteins fused to the C-terminus of the EGFP protein. HEp-2 cells were grown to 80% confluent monolayers by standard tissue culture techniques in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc.) containing 10% fetal calf serum (FCS). For immunoprecipitation, approximately 10 x $10^6$ cells were transfected with 20-30 μg of DNA in 1600 μl of DMEM containing 10% FCS by electroporation, which was performed at 270V and 950 μF using a Gene-Pulser II (Bio-Rad). After transfection, cells were seeded in 75-$cm^2$ culture flasks and cultured overnight. After washing twice with PBS, the cells were resuspended in 500 μl of lysis buffer (25 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM EDTA, 1 mM DTE, 0.5 mM PMSF and 0.05% NP-40) and homogenized by sonification. For fluorescence microscopy, approximately 2 x $10^6$ cells were transfected with 10-20 μg of DNA in 800 μl of DMEM containing 10% FCS by electroporation, as described above. After transfection, cells were seeded onto coverslips and cultured overnight. Cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, washed in PBS twice and finally cells were mounted with PBS/glycerol, and expressed proteins were visualized by fluorescence microscopy.

### *Immunoprecipitation*

Polyclonal antibodies from rabbits and patients were coupled to protein A-agarose beads (Biozym) in IPP500 (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40) by incubation for 2 h at room temperature. Beads were washed twice with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40). For each immunoprecipitation, cell extract was incubated with the antibody-coupled beads for 1 h at 4°C. Subsequently, beads were washed three times with IPP150.

### *Mammalian two-hybrid analysis*

All interactions were analyzed using the CheckMate Mammalian Two-Hybrid System (Promega) essentially according to the manufacturer's protocol. Briefly, 3-4 x $10^5$ COS-1 cells seeded in one well of a 6-wells plate were transfected with 3 vectors (1 μg each), pACT and pBIND, either with or without insert, and the pG5*luc* reporter vector using 5 μl Fugene Transfection Reagent (Roche), as described by the manufacturer. After 40-48 hours of growth, cells were harvested using 500 μl Passive Lysis Buffer (PLB; Promega) and the activity of both the firefly luciferase and the control renilla luciferase were determined using the Dual Luciferase Reporter Assay System (Promega) on a Berthold Lumat LB 9507 Luminometer. In brief, 100 μl of firefly luciferase substrate solution was added to 20 μl of cell extract and the luminescence was measured to determine the efficiency of the interaction. Next, 100 μl of

renilla luciferase substrate solution (containing a quencher for the firefly luciferase activity) was added and again the luminescence was determined to monitor the transfection efficiency.

*Expression of recombinant proteins*

The GST (fusion) proteins were expressed in bacteria and purified as described previously (87). After induction with IPTG the bacteria expressing the GST-hCsl4p fusion protein were grown at room temperature to enhance the production of soluble protein, whereas the cells producing GST were grown at 37°C.

In vitro *translation*

Radioactively labeled proteins were produced using the TnT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocol, in a total volume of 25 µl, containing approximately 1 µg of circular plasmid (pCDNA3, Invitrogen) containing the coding sequence of hRrp42p or hRrp46p or another protein and in the presence of [$^{35}$S]methionine.

*GST pull-down assay*

Recombinant GST or GST-hCsl4p fusion protein (approx. 1 µg) immobilized on glutathione-Sepharose beads (Amersham Pharmacia Biotech) was incubated with [$^{35}$S]methionine-labeled in vitro translation product in 200 µl of binding buffer (20 mM HEPES-KOH pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTE, 0.5 mM EDTA, 0.05% NP-40, 0.02% BSA, Complete Protease Inhibitor Cocktail (Roche)). The mixture was incubated for 2 hours at 4°C on a rotator, after which the beads were washed three times with wash buffer (20 mM HEPES-KOH pH 7.9, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTE, 0.5 mM EDTA, 0.05% NP-40, 0.02% BSA, Complete Protease Inhibitor Cocktail (Roche)). After removing the supernatant in the final wash, samples were resuspended in 20 µl of 2× SDS sample buffer and boiled for 5 minutes. Finally, samples were resolved by 10% SDS-PAGE and visualized by autoradiography.

*Exoribonuclease activity assay*

Immunoprecipitations were performed as described above. After removal of non-bound material, immune complexes bound to the protein A-agarose beads were washed twice with buffer A (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$ ,1 mM $Na_2HPO_4$ ). Substrate solution (approx. 100 ng $^{32}$P-labeled substrate in 40 µl buffer A) was added to the protein A beads, and the suspension was incubated at 37 °C with gentle agitation. Formamide loading buffer was added to 10 µl samples taken at regular intervals and these were immediately frozen until analysis. Samples were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. Substrates, transcribed in vitro by T3 RNA polymerase from XbaI-linearized pBS(-) template (Stratagene), were labeled randomly using [$^{32}$P]UTP (Amersham Pharmacia Biotech).

# Chapter 3

## Protein-protein interactions between human exosome components support the assembly of RNase PH-type subunits into a six-membered PNPase-like ring

Reinout Raijmakers
Wilma Vree Egberts
Walther J. van Venrooij
Ger J.M. Pruijn

*Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, Nijmegen, The Netherlands*

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The exosome is a complex of 3′ → 5′ exoribonucleases, which functions in a variety of cellular processes, all requiring the processing or degradation of RNA. Here we present a model for the assembly of the six human RNase PH-like exosome subunits into a hexameric ring structure. In part this structure is based upon the evolutionarily related bacterial degradosome, the core of which consists of three copies of the PNPase protein, each containing two RNase PH domains. In our model three additional exosome subunits, which contain S1 RNA binding domains, are positioned on the outer surface of this ring. Evidence for this model was obtained by the identification of protein-protein interactions between individual exosome subunits in a mammalian two-hybrid system. In addition, the results of co-immunoprecipitation assays indicate that at least two copies of hRrp4p and hRrp41p are associated with a single exosome, strongly suggesting that at least two of these ring structures are stacked in this complex. Finally, the identification of a human gene encoding the putative human counterpart of the bacterial PNPase protein is described, which suggests that the exosome is not the eukaryotic equivalent of the bacterial degradosome, although they do share similar functional activities.

## Introduction

Many cellular events in eukaryotic cells that include 3'→5' processing or degradation of RNA require the activity of a multi-protein complex called the exosome. Among these processes are the maturation of 5.8S rRNA(12,31,32), the processing of many small RNAs (33,38,42) and the turnover of different types of mRNAs (17,57,82,83). The exosome consists of at least 11 proteins, all of which have been either shown to possess 3'→5' exoribonuclease activity or are predicted to contain exoribonuclease or RNA binding activity based on their homology with prokaryotic proteins(10-12). These prokaryotic proteins include RNase PH, RNase R, RNase D and the RNA binding domain of the S1 ribosomal protein.

Ten of the eleven human proteins that were identified as putative exosome subunits have been shown to be associated with the human exosome complex (14,17). These include the polymyositis/scleroderma overlap syndrome autoantigens PM/Scl-75, PM/Scl-100 and hRrp4p (78,79).

Our current knowledge on the interactions between different exosome subunits and on the overall structure of the exosome is limited. Recently, we have demonstrated that the hCsl4p protein binds to two other human exosome subunits, hRrp42p and hRrp46p (88). In genome-wide yeast two-hybrid screens interactions between the *S. cerevisiae* Mtr3p and Rrp42p and between Rrp41p and Rrp45p proteins have been identified (67,85). Finally, in *Arabidopsis thaliana* AtRrp4p and AtRrp41p display a direct interaction (26), whereas AtRrp41p is able to interact with the yeast Rrp45p and Rrp44p proteins (22).

In eubacteria and chloroplasts, 3'→5' RNA decay is carried out by a multiprotein complex called the degradosome (89,90). The bacterial degradosome contains several proteins, including the endoribonuclease RNase E, the exoribonuclease polynucleotide phosphorylase (PNPase), enolase (a glycolytic enzyme) and an RNA

helicase, termed RhlB (91,92). The chloroplast degradosome contains only PNPase and no other proteins (90).

The structure and assembly of the bacterial degradosome has been studied in detail and RNase E was found to play a key role in the structural organization of this complex (93). In addition, the crystal structures of the degradosome components PNPase and enolase have been resolved (94,95). Interestingly, PNPase is a tightly associated trimeric enzyme (20), with the six RNase PH domains (RPDs) arranged in a ring-like structure. Very recently, the EM-structure of the yeast exosome has been determined and appeared to be consistent with six RPD containing exosome proteins arranged in a hexameric ring and three S1/KH proteins associated with the outer surface of the ring (21).

In this study we systematically analysed mutual interactions between all known subunits of the human exosome in a mammalian two-hybrid system. In combination with protein module similarities between human exosome subunits and the bacterial PNPase, our data were used to generate a model for the human exosome, the main feature of which is a ring composed by the six RNase PH type subunits, which is consistent with the EM-structure of the yeast exosome.

## Results

### *Interactions between human exosome subunits*

To identify protein-protein interactions in the human exosome, the full-length cDNAs encoding 11 (putative) human exosome components (hRrp4p, hRrp40p, hRrp41p, hRrp42p, hRrp46p, PM/Scl-75, PM/Scl-100, hCsl4p, hRrp44p/hDis3p, OIP2 and hMtr3p) were cloned in both the pACT (in-frame with the sequence encoding the VP16 transcription activation domain) and pBIND (in-frame with the sequence encoding the GAL4 DNA binding domain) vectors of the Checkmate Mammalian Two-Hybrid system. COS-1 cells were co-transfected with each pair of these constructs and after 40-48 hours the luciferase activity of extracts prepared from these cells was determined. The expression levels of the fusion proteins were monitored by western blot analysis using antibodies specific for the VP16 or GAL4 (Santa Cruz) domains. This showed that all

| pBIND \ pACT | hRrp4p | hRrp40p | hRrp41p | hRrp42p | hRrp46p | PM/Scl-75 | PM/Scl-100 | hCsl4p | hDis3p | OIP2 | hMtr3p | control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hRrp4p | 39 | 41 | 472 | 2657 | 33 | 33 | 25 | 35 | 34 | 33 | 39 | 26 |
| hRrp40p | 56 | 6 | 15 | 21 | 68 | 20 | 13 | 24 | 10 | 21 | 22 | 18 |
| hRrp41p | 141 | 32 | 84 | 36 | 10 | 32 | 44 | 58 | 25 | 7 | 112 | 16 |
| hRrp42p | 3831 | 25 | 142 | 28 | 14 | 34 | 30 | 100 | 36 | 14 | 991 | 17 |
| hRrp46p | 23 | 24 | 19 | 13 | 11 | 14 | 13 | 103 | 17 | 83 | 41 | 16 |
| PM/Scl-75 | 25 | 33 | 14 | 17 | 9 | 28 | 20 | 30 | 25 | 8 | 17 | 13 |
| PM/Scl-100 | 39 | 17 | 36 | 18 | 14 | 42 | 18 | 26 | 21 | 12 | 21 | 15 |
| hCsl4p | 95 | 59 | 146 | 151 | 298 | 121 | 60 | 16 | 83 | 20 | 430 | 52 |
| hDis3p | 16 | 11 | 19 | 15 | 3 | 19 | 17 | 31 | 18 | 6 | 6 | 1 |
| OIP2 | 41 | 14 | 52 | 26 | 135 | 37 | 27 | 29 | 25 | 15 | 786 | 31 |
| hMtr3p | 35 | 17 | 11 | 325 | 17 | 7 | 9 | 89 | 9 | 126 | 101 | 4 |
| control | 74 | 49 | 36 | 78 | 18 | 53 | 52 | 53 | 47 | 30 | 101 | 30 |

**Figure 1. Summary of protein-protein interaction data for human exosome components as determined in a mammalian two-hybrid system.**
COS-1 cells were cotransfected in a pairwise manner with constructs encoding the human exosome subunits fused to either the VP16 transcription activation domain (pACT constructs) or the GAL4 DNA binding domain (pBIND constructs), in combination with a luciferase encoding reporter plasmid. The activation of luciferase expression resulting from two-hybrid interactions is expressed in relative luminescence units (RLU). An interaction was considered positive when the RLU exceeded the sum of the two corresponding control measurements (the luciferase activity observed when the constructs were co-transfected with either the 'empty' pACT or the 'empty' pBIND vector, shown at the right and at the bottom). The activity observed for the combination of hRrp42p in pBIND and hCsl4p in pACT was defined as 100 RLU. Interactions observed in both of the two possible combinations are shaded dark gray, whereas interactions observed in one direction only are shaded light gray.

proteins were expressed at comparable levels (data not shown). An overview of the results is given in Figure 1. Eight interactions were observed in both pairwise combinations. In addition, one self-interaction was identified (hRrp41p) and four interactions were detected in one direction only. Two of these four, however, include the hCsl4p/pBIND construct, which gives rise to a relatively high background value in combination with the 'empty' pACT vector (Fig. 1). Because the assay was carried out using primate cells (COS-1), there is a possibility that certain interactions are mediated by endogenous (exosome) proteins. Previously, however, some of the interactions identified in this system (hRrp42p with hCsl4p and hRrp46p with hCsl4p) were confirmed by GST-pulldown experiments (88).

### *Homology between PNPase and the human exosome*

In the PNPase proteins of most prokaryotes (including *E. coli* and *Streptomyces antibioticus*) 5 distinct domains can be discerned: 2 RPDs, an α-helical domain, a KH domain and a domain showing homology with the S1 RNA binding domain (94). Six human exosome subunits (hRrp41p, hRrp42p, hRrp46p, PM/Scl-75, OIP2 and hMtr3p) also share homology with RNase PH and three others (hRrp4p, hRrp40p and hCsl4p) contain a putative S1 RNA binding domain (11,17). Moreover, the recently characterized *A. thaliana* Rrp4p protein contains a KH domain, capable of binding RNA, which can also be discerned in the yeast and human Rrp4p and Rrp40p proteins(26). These similarities suggest that there is structural homology between the exosome and the PNPase protein complex (20,21).

To identify with which of the two RPDs of PNPase the human and yeast exosome proteins that contain an RPD share the highest degree of homology, the RPDs of all exosomal RNase PH-type subunits were compared with the RPDs of a number of PNPases from different organisms. Figure 2A shows a multiple alignment of the segment of these RPDs that is most conserved among PNPases and exosome components. This shows that the second RPD of PNPase is more conserved than the first and that a clear distinction can be made between the two RPDs of each PNPase. Strikingly, the RPDs of three human (hRrp41p, hRrp46p and hMtr3p) and the corresponding three yeast exosome proteins displayed the highest degree of sequence homology with the second RPD of PNPases, whereas the other three human RNase PH-type exosome subunits (hRrp42p, PM/Scl-75 and OIP2) and the corresponding yeast counterparts appeared to be more homologous to the first RPD of PNPases. To demonstrate that the same grouping of RPD containing exosome subunits was obtained when the sequence of the complete RPDs was taken into account a cladogram was generated using a multiple sequence alignment of the RPD sequences defined in the Pfam database (http://pfam.wustl.edu). The results confirm the grouping of RPD containing exosome subunits described above based upon their homology with the two RPDs of PNPases. Only a single exosome subunit, the yeast Mtr3p, appeared to fall in the other group by this analysis. However, we believe that the yeast Mtr3p belongs to the RPD2 group based upon its homology with its human counterpart hMtr3p and the presence of a RPD2 signature sequence (LALXDAG) in its most highly conserved element (see Figure 2A).

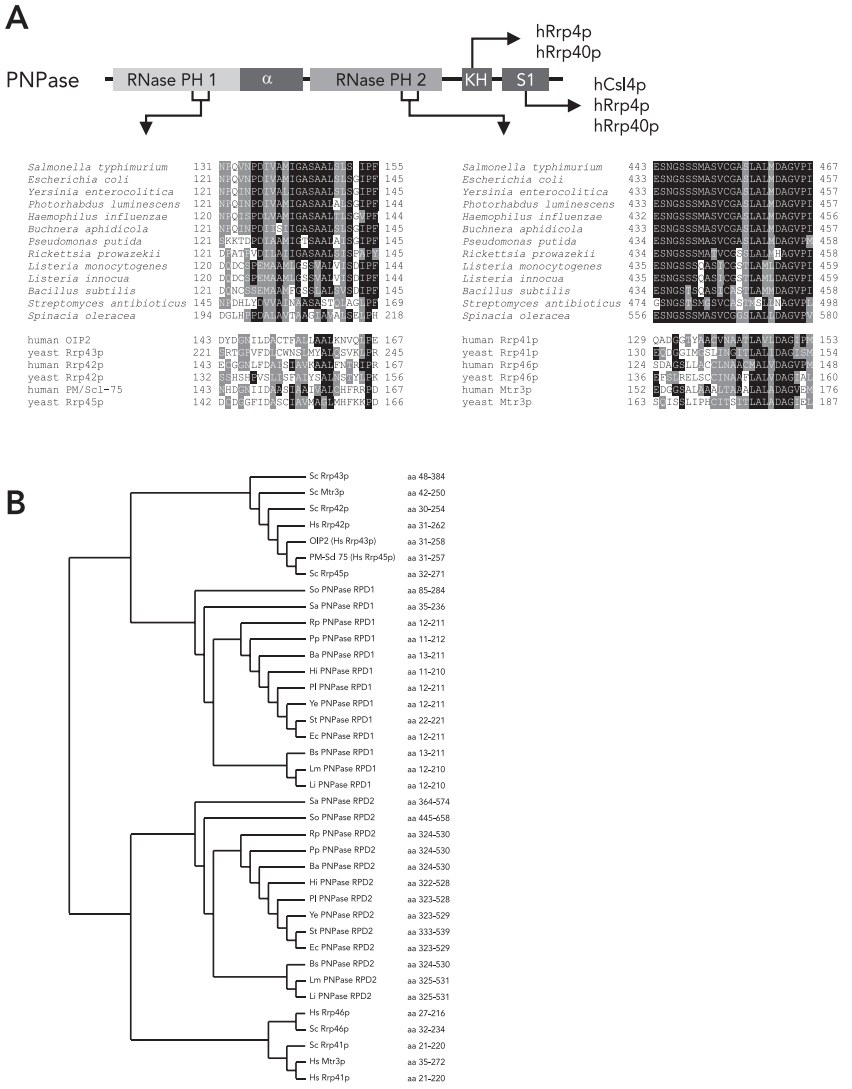


**Figure 2. Sequence homology between bacterial PNPase and the human and yeast exosome subunits.**
A. The relative positions of the five protein domains that can be discerned in a typical bacterial PNPase protein are schematically depicted in the upper part of the Figure. The exosome proteins that share homology with these domains are indicated. The lower part of the figure shows sequence alignments of the segment of the RPDs that is most highly conserved among the PNPase and exosome proteins. The sequences of 12 prokaryotic and 1 plant (*Spinacia oleracea*) PNPase proteins are compared with the human and yeast RNase PH-type exosome subunits. Numbers indicated the positions of the first and last amino acid of these segments in the respective polypeptides. Black-boxed amino acids are identical in more than 50% of the sequences and grey boxes indicate conservation of similar amino acids.
B. Cladogram based upon a multiple sequence alignment (generated by ClustalW) of the complete RPDs of the proteins shown in A. Sc, *S. cerevisiae*; Hs, *H. sapiens;* So, *S. oleracea*; Sa, *S. antibioticus*; Rp, *Rickettsia prowazekii*; Pp, *Pseudomonas putida*; Ba, *Buchnera aphidicola*; Hi, *Haeomophilus influenza*; Pl, *Photorhabdus luminescens*; Ye, *Yersinia enterocolitica*; St, *Salmonella typhimurium*; Ec, *E. coli*; Bs, *B. subtilus*; Lm, *Listeria monocytogenes*; Li, *Listeria innocua*. RPD1 and RPD2 refer to the most N-terminal and most C-terminal RPD, respectively. The boundaries of the RPDs (indicated by numbers of amino acid positions) were identified using the Pfam database which is based on Hidden Markov Model searches.

*Model of the human exosome*

The striking similarity of protein domains present in exosome subunits on the one hand and the PNPase entity of the degradosome on the other hand, as well as their functional relationship suggested that their architecture might also be similar.

The structure of *S. antibioticus* PNPase as obtained by X-ray crystallography has revealed that this protein exists as a homo-trimeric complex, in which the six RPDs form a ring-shaped hexameric structure. The S1 and KH RNA binding domains are positioned at the periphery of this ring, which may facilitate the recruitment of the RNA substrates (94).

Based upon the PNPase structure and taking into account the observed interactions between different exosome subunits (Figure 1), a model for the human exosome was generated in which the six RNase PH-like subunits form a hexameric ring and the three proteins with S1 (and KH) RNA binding domains are positioned at the outer surface of this ring. A graphical representation of this model is depicted in Figure 3. The two-way interaction between hRrp46p and hCsl4p is omitted from this model and may only occur in vitro due to sequence or structural homology between hRrp46p and hMtr3p. In agreement with the *S. antibioticus* PNPase structure the ring-shaped hexamer is predicted to be comprised of three pairs of antiparallel domains. Based upon the presently available data it is hardly possible to predict which are the most closely interacting pairs around the ring, i.e. two exosome subunits that correspond to a single PNPase moiety. The analysis of mutants of hRrp46p, lacking either the N- or the C-terminus of the protein, demonstrated that the C-terminal part of hRrp46p interacts with OIP2 (data not shown). In PNPase the C-terminal regions of the RPDs mediate the intramolecular interaction between the two RPDs (94). This suggests that hRrp46p and OIP2 correspond to one PNPase moiety in the ring structure, and extrapolation would mean that PM/Scl-75 – hRrp41p and hRrp42p – hMtr3p represent the other pairs.

The proposed ring structure for the exosome is expected to contain a single copy of all RNase PH- and S1-like subunits. If, however, two or more of such rings assemble into a larger com-

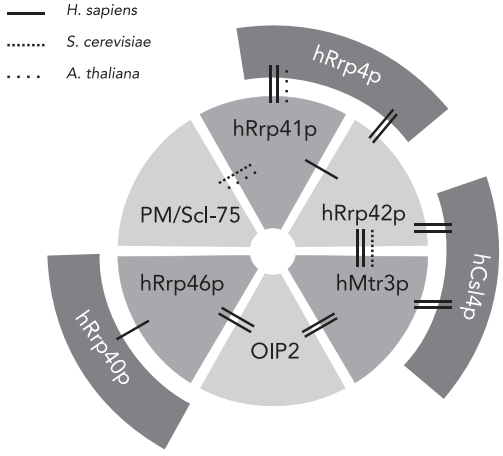


**Figure 3. Structural model for the human exosome.**
This model is based on the protein-protein interactions identified by two-hybrid analysis and the structure of bacterial PNPase. Shading of the proteins indicates their homology with the RPDs 1 (light gray, black letters) and 2 (gray, black letters) of bacterial PNPase and the S1 and KH RNA binding domains (dark gray, white letters). Interactions between human exosome subunits are indicated by solid lines (interactions detected in the two-hybrid system in two directions with double lines) and interactions reported for the exosome subunit counterparts of either *S. cerevisiae* or *A. thaliana* are indicated by dotted lines.

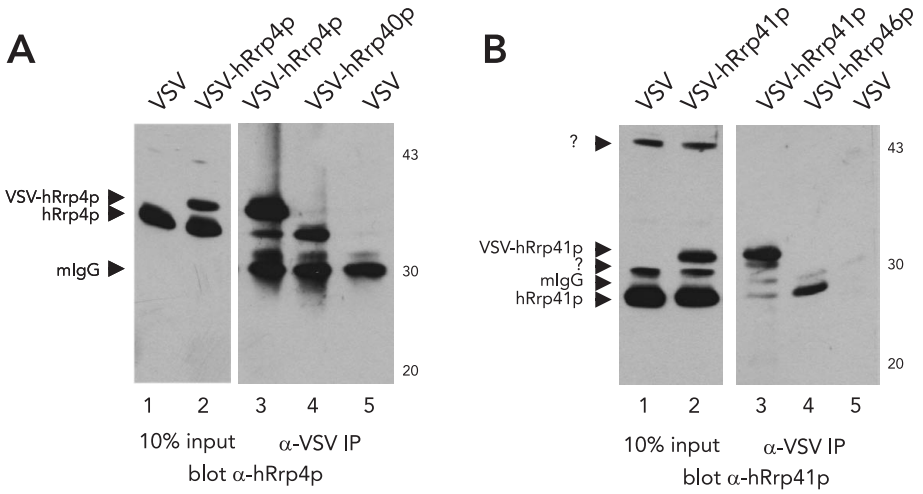


**Figure 4. At least two copies of exosome components reside in a single complex.**
VSV-G-tagged hRrp4p, hRrp40p, hRrp41p and hRrp46p were expressed in transiently transfected HEp-2 cells and subsequently cell lysates were subjected to immunoprecipitation with anti-VSV-G-tag antibodies (α-VSV IP). Co-precipitated proteins were analyzed by western blotting using anti-hRrp4p (A) and anti-hRrp41p (B) antibodies. In both panels, lanes 1 and 2 contain 10% of the extracts from cells expressing only the VSV-G-tag (lanes 1), the VSV-G-tagged hRrp4p (A, lane 2) or hRrp41p (B, lane 2) that were used for immunoprecipitation. Immunoprecipitated proteins from the extracts containing VSV-G-hRrp4p (A, lane 3), VSV-G-hRrp40p (A, lane 4), VSV-G-hRrp41p (B, lane 3), VSV-G-hRrp46p (B, lane 4), or only the VSV-G-tag (A and B, lane 5) were analyzed in lanes 3-5. The positions of (VSV-G-tagged) hRrp4p and hRrp41p are indicated on the left of each panel; the positions of molecular mass markers on the right (in kDa). The extra bands visible in lanes 3-5 (marked mIgG) are due to cross-reactivity of the rabbit sera with the mouse anti-VSV-G-tag IgG used for the immunoprecipitations and unidentified proteins in total extracts that are recognized by the anti-hRrp41p rabbit serum (panel B, lanes 1 and 2) are marked by "?".

plex, as has been observed for other eukaryotic multiprotein complexes that assemble into ring-like structures (e.g. the proteasome and the Hsp60 complex), at least two copies of each ring component would reside in the complex. To investigate these possibilities, we expressed tagged versions of two human exosome components in transfected cells and analyzed whether the tagged protein and the non-tagged endogenous counterpart associated with a single exosome complex. HEp-2 cells were transfected with constructs encoding either VSV-G-tagged hRrp4p (S1-type) or VSV-G-tagged hRrp41p (RNase PH-type) and from these cells lysates were prepared and subjected to immunoprecipitations with anti-VSV-G-tag monoclonal antibodies. As a positive control for the precipitation of endogenous hRrp4p and hRrp41p, parallel transfections were performed with constructs encoding VSV-G-tagged hRrp40p and hRrp46p. The immunoprecipitates were analyzed by western blotting using rabbit sera directed to either hRrp4p or hRrp41p. The results in Figure 4 show that for both of the proteins analyzed co-precipitation of the non-tagged endogenous counterpart was indeed observed (lanes 3). Importantly, no co-precipitation of the endogenous proteins was detectable when lysates from cells transfected with constructs encoding the VSV-G tag only were used (lanes 5) and as expected hRrp4p and hRrp41p were efficiently coprecipitated from lysates prepared from cells expressing VSV-G tagged hRrp40p and hRrp46p, respectively (lanes 4). These data suggest that at least two copies of hRrp4p and hRrp41p are

present in the assembled exosome. Because both of these proteins are part of the proposed exosome ring structure, two or more of these rings may be present in one complex. The relatively weak signals for the endogenous proteins in the corresponding anti-VSV immunoprecipitates may be due to overexpression of the VSV-tagged proteins in transiently transfected cells in combination the use of anti-VSV-G-tag antibodies (see Discussion).

### *Identification of human PNPase*

The structural similarity between the *S. antibioticus* PNPase and the exosome raised the question whether the exosome represents the eukaryotic counterpart of the eubacterial PNPase/degradosome. Alternatively, eukaryotes may also express a PNPase/degradosome and both complexes might have a similar evolutionary origin. The latter option is supported by the identification of a PNPase containing degradosome in chloroplasts of *Spinacia oleracea*. To shed more light on this issue, several eukaryotic genome databases were screened for the presence of genes homologous to *S. antibioticus* PNPase. This resulted in the detection of PNPase homologues in *C. elegans* and *D. melanogaster*. The *C. elegans* (acc. no. BE0003N10.1) and the *D. melanogaster* (acc. no. CG11337) amino acid sequences are 26% and 28% identical (38% and 40% similar), respectively, to the *S. antibioticus* PNPase sequence. Next, the human genome was screened with the

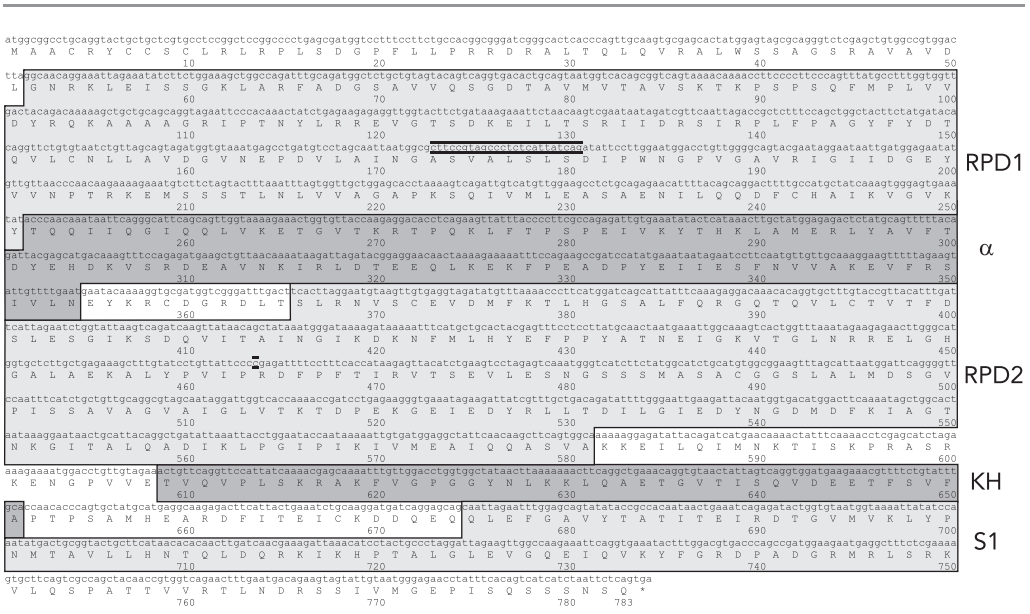


**Figure 5. Sequence and modular structure of human PNPase.**
The nucleotide sequence of the coding region of human PNPase was composed as described in the text using a combination of genomic and EST database entries. The derived amino acid sequence is shown below the nucleotide sequence. Numbering refers to the amino acid sequence. The positions of protein domains found in all identified PNPases are indicated: two RPDs (1 and 2), separated by an α-helical region, a KH domain and an S1 RNA binding domain. The coding region is 2352 nucleotides long, corresponding to 783 amino acids. Nucleotides that were found to be different in the cDNA obtained from the cDNA library are indicated with bars below and above these nucleotides. The DNA and protein sequence shown here have been submitted to the EMBL database (accession number AJ458465).

PNPase-like polypeptide sequences of *C. elegans* and *D. melanogaster*. This led to the detection of a sequence (LOC87178) on chromosome 2 that is homologous to the C-terminal part of these PNPase-like polypeptides. Both a putative S1 RNA binding domain and a putative KH domain are present in the polypeptide encoded by this genomic sequence, as demonstrated by a screening using the Pfam protein families database (96). Screening of the human EST (expressed sequence tag) databases with this sequence allowed the identification of the N-terminal part of this polypeptide, which encompasses a putative mitochondrial targeting sequence and two putative RPDs (identified by Pfam) separated by a predicted alpha-helical region (identified by Jpred (97)). The annotated genomic sequence comprises only the C-terminal part of the complete polypeptide, which is most likely due to an extra guanosine residue in the genomic sequence at position 1739 (adenosine of startcodon is numbered 1). This additional residue is not present in the EST sequences corresponding to this region and thus is likely to be a sequencing error. The complete coding sequence and the derived polypeptide sequence of the putative human PNPase protein, as well as its modular structure, are shown in Figure 5. Based upon the available EST sequences the 5' and 3' untranslated regions (UTR) of the putative human PNPase mRNA are predicted to be 26 and 118 nucleotides long, respectively. The putative human PNPase amino acid sequence is 36% identical (49% similar) to *E. coli* PNPase, 29% identical (49% similar) to *S. antibioticus* PNPase, 37% identical (51% similar) to *C. elegans* PNPase and 51% identical (64% similar) to *D. melanogaster* PNPase. A comparison of the putative human PNPase polypeptide sequence with that of the human exosome proteins revealed no significant homology other than the residues that are also conserved between the human exosome components and the prokaryotic PNPase proteins.

The three eukaryotic PNPase-like sequences described above were used to screen the *S. cerevisiae* and *M. musculus* genomes for a similar sequence, but no gene encoding a putative PNPase could be identified for these organisms. The mouse EST database, however, contains a number of EST sequences encoding a putative murine PNPase homologue, substantiating the existence of PNPase in higher eukaryotes.

Although the human and mouse ESTs corresponding to the PNPase sequences indicate that the genes encoding this polypeptide are indeed transcribed in these organisms, we wanted to have more convincing evidence that the human PNPase sequence derived from the database entries is indeed expressed in humans. Therefore, human a cDNA library was used to isolate a complete PNPase cDNA with primers derived from the predicted sequence by PCR. PCR were products obtained from a teratocarcinoma cDNA library, the sequence of which appeared to be almost identical to that of the predicted human PNPase sequence. The only differences observed were a deletion of nucleotides 518-541 (adenosine of startcodon is numbered 1) and a single nucleotide substitution at position 1390. The absence of nucleotides 518-541, is likely to be due to an alternative splicing event and the substitution of nucleotide 1390, which does not change the encoded amino acid, may be a polymorphism. Although the single nucleotide substition is also represented in the EST databases, all EST sequences of that region of the PNPase mRNA do contain nucleotides 518-541.

## Discussion

In this paper we propose a structural model of the human exosome based on the homology between the exosome and bacterial PNPase, the three-dimensional structure of which has been solved by X-ray crystallography. This model is strongly supported by a number of interactions between human exosome components, which were identified by mammalian two-hybrid analyses. In addition, database searches led to the identification of a sequence that appears to encode the human orthologue of the PNPase protein. The latter finding suggests that humans, and possibly also other eukaryotes, express two exoribonuclease complexes that are structurally similar to the bacterial PNPase, the exosome and the putative human PNPase.

### *Interactions between exosome components*

Most of the interactions between human exosome subunits identified by mammalian two-hybrid analysis are in agreement with the model presented for the human exosome. The only human protein which did not detectably interact with other subunits in this system is the PM/Scl-75 protein. In one of two extensive yeast two-hybrid screens, however, its yeast homologue Rrp45p was shown to interact with yeast Rrp41p (85), the homologue of hRrp41p, which is indeed interacting with PM/Scl-75 in the model shown in Figure 3. The inability of PM/Scl-75 to interact in vitro with other proteins might point to an important role of the N-terminus of this protein, which is fused to either the transcription activation or the DNA-binding domain in the two-hybrid analysis and thus might be functionally impaired. This is substantiated by observations made with N- and C-terminal EGFP-fusion proteins of PM/Scl-75, which were unable to enter the nucleolus (unpublished observations), in contrast to other EGFP-tagged exosome subunits and to the endogenous PM/Scl-75 (8,88). Moreover, the yeast protein Rrp45p has been shown to bind *in vitro* to GST-tagged *A. thaliana* Rrp41p, but this interaction was lost when a C-terminal hexahistidine tag was attached to Rrp45p (22). Taken together, these data suggest that both termini of PM/Scl-75 and Rrp45p are involved in their interaction with other exosome subunits.

Four of the observed two-hybrid interactions are not explained by the model we generated. These include the interactions of hCsl4p with hRrp41p and PM/Scl-75, both of which were detected only with the hCsl4p/pBIND construct, which gives rise to relatively high background signals, and the interaction of hRrp41p with itself. The fourth two-hybrid interaction our model does not account for is that of hCsl4p with hRrp46p. This interaction may be explained by structural homology between hMtr3p and hRrp46p (sequence identity 25%, similarity 36%), which are both homologous to the second RPD of PNPase. This possibility is supported by the observation that hMtr3p binds to the same region of hCsl4p as does hRrp46p (data not shown) (88).

The model for the human exosome complex proposed here (a PNPase-like ring) is expected to be applicable to the exosome of other organisms as well, including yeast. Interestingly, the very recently reported EM-structure for the yeast exosome fully supports the arrangement of the RPD containing subunits in a hexameric ring (21). These authors have predicted the relative position of the RPD-type subunits in the ring based upon bioinformatics data and this aspect of their model only partially agrees with our

model for the human exosome. Although the discrepancy might reflect species differences, it is more likely due to an alternative interpretation of the homologies among the exosome subunit RPDs. Based on a predicted functional site of 5 amino acids (which is in fact located just outside the approximately 200 amino acid long RPD as predicted by Pfam), the yeast exosomal RPD proteins were classified as homologues of either RPD1 (Rrp43, Rrp46, Mtr3) or RPD2 (Rrp41, Rrp42, Rrp45) of PNPase. When the complete RPDs (as predicted by Pfam) from many exosome and PNPase proteins or the most highly conserved segment of these are used for the analysis, Rrp42, Rrp43 and Rrp45 appear to be more closely related to RPD1 and Rrp41, Rrp46 and Mtr3 appear to be more closely related to RPD2 (Figure 2). Importantly, the arrangement of the RPD subunits in our model is strongly supported by the available protein-protein interaction data for both the yeast and the human exosome. The only two interactions identified between yeast exosome components (Mtr3p with Rrp42p and Rrp41p with Rrp45p) are consistent with our model (Figure 3), but not with the model proposed by Aloy *et al*. (21).

Two other human exosome components, PM/Scl-100 and hDis3p are not incorporated in the model since they were unable to interact *in vitro* with other exosome subunits and no significant sequence homology between these proteins and any component of the bacterial degradosome could be identified. Currently, no evidence is available for the association of hDis3p with the human exosome, except for its homology with the yeast exosome protein hRrp44p. The recent purification and sequencing of all human exosome proteins confirmed the association of most putative human exosome proteins, including PM/Scl-100, but hDis3p was not detected (17). PM/Scl-100 shares homology with the bacterial exoribonuclease RNase D (11), which is not associated with the degradosome. PM/Scl-100 and its yeast homologue Rrp6p have been suggested to be exclusively associated with the nuclear exosome complex, suggesting that it is indeed not a core component of the exosome (11). This is supported by the results of size-exclusion chromatography experiments, which demonstrated that PM/Scl-100 is only associated with the higher molecular mass exosome complexes (approx. 700 kDa) but not with lower molecular mass complexes (250 - 600 kDa), in contrast to four putative core components, which were detected in all complexes (14). The failure of PM/Scl-100 to interact with other exosome components in the mammalian two-hybrid system might be due to the fact that this protein requires more than one of the core components for efficient binding.

The results of the co-immunoprecipitation experiments suggest that (part of) the exosome complexes contain at least two copies of hRrp4p and hRrp41p. The relatively low level of co-precipitation of the endogenous, non-tagged, hRrp4p and hRrp41p proteins was expected in this experimental setting. An excess of a VSV-tagged subunit in a transfected cell may allow only part of it to become incorporated in exosome complexes, whereas both 'free' and exosome associated VSV-tagged protein will be precipitated. Moreover, not all exosome complexes in a cell may be composed of two rings. An alternative explanation for these data, which may be unlikely but can not be completely ruled out yet, is the presence of more than one copy of hRrp4p and hRrp41p in a single ring. The observation that more than one copy of the pre-

sumptive core components and thus more than one ring may be present in the same exosome complex raises the question what the overall structure of the exosome is. One option is that the exosome forms a proteasome- or Hsp60-like structure (which consist of 4 or 2 stacked heptameric rings, respectively(98,99)) by direct ring-ring interactions. Another possibility is that PM/Scl-100 and/or hDis3p are able to associate with more than one ring simultaneously, thereby combining two or more rings into one complex. Interestingly, the calculated molecular mass of a complex containing one copy of each of the 'core' subunits of the human exosome (Figure 3) is approx. 260 kDa, which means that a complex consisting of two rings and one copy of PM/Scl-100 and hDis3p (both approx. 100 kDa) would indeed be around 700 kDa, in good agreement with the size-exclusion chromatography data (14). The appearance of 250-600 kDa complexes may represent disassembled or only partially assembled exosomes. Based upon its EM-structure, the yeast exosome does not appear to consist of multiple rings (21), which is in agreement with its molecular mass of 300-400 kDa (12). This may reflect an important structural difference between the human and yeast exosome complexes. The purification of native human exosome complexes and subsequent structural analysis by (cryo-)EM will be required to shed more light on this issue.

### *The exosome versus PNPase*

The recently identified structural conservation between the prokaryotic PNPase and the eukaryotic exosome suggests that these two complexes function by similar mechanisms (20). Both have been reported to be involved in the degradation of mRNA and rRNA. Although the functions of both complexes seem to be redundant, in a number of eukaryotic organisms, including human, both complexes appear to exist. The presence of PNPase in chloroplasts has already been described previously (90). Chloroplasts (and also mitochondria) have been suggested to originate from eubacteria that were taken up by primitive eukaryotes. Many eubacteria are known to contain PNPase proteins (10), whereas archaebacteria have been shown to contain exosome components (86), but no PNPase homologues. These data suggest that the eukaryotic exosome has evolved from the archaeal exosome proteins, whereas the eukaryotic PNPase stems from a eubacterial protein. Whether the human PNPase, although encoded by a nuclear gene, is specific for mitochondria as is the plant PNPase for chloroplasts remains to be investigated. Remarkably, all three animal PNPase proteins identified (human, *D. melanogaster* and *C. elegans*) contain an extended N-terminal sequence in comparison with the bacterial PNPases, which varies from 51 amino acids in human to 33 in *C. elegans* (with respect to the most N-terminal RPD). In all three cases, this sequence is enriched in hydrophobic, hydroxylated and positively charged amino acids and therefore might serve as a mitochondrial import signal, similar to that of known nuclear encoded mitochondrial proteins (100). If the putative human PNPase protein indeed appears to reside in the mitochondria, this protein may function in RNA processing/decay processes that are specific for this organelle, whereas the exosome performs similar functions in the cytoplasm, nucleoplasm and nucleolus of cells.

## Materials and Methods

### *cDNA cloning*

All cDNAs used were cloned into the pACT and pBIND vectors (Promega). The database accession numbers of the cDNAs used are BC000747 (hRrp4p), AF281132 (hRrp40p), AF281133 (hRrp41p), D29958 (hRrp42p), AF281134 (hRrp46p), M58460 (PM/Scl-75), L01457 (PM/Scl-100), AF151866 (hCsl4p), R27667 (hDis3p), AF025438 (OIP2) and NM_058219 (hMtr3p). The cDNA encoding the putative human PNPase was submitted under accession number AJ458465.

### *Mammalian two-hybrid analysis*

All interactions were analyzed using the CheckMate Mammalian Two-Hybrid System (Promega). Briefly, approx. 3.5 x $10^5$ COS-1 cells were transfected with 3 vectors (1 µg each), pACT and pBIND, either with or without insert, and the pG5*luc* reporter vector using 5 µl Fugene Transfection Reagent (Roche). After culturing for 40-48 hours, cells were harvested using 500 µl Passive Lysis Buffer (PLB; Promega) and the expression of both the firefly luciferase and the control Renilla luciferase were quantified using the Dual Luciferase Reporter Assay System (Promega) on a Berthold Lumat LB 9507 Luminometer.

### *Bioinformatics*

Multiple alignments of PNPase and exosome protein sequences were generated with ClustalW, using BLOSUM30 substitution matrices. Where necessary, the multiple alignments obtained were corrected manually. Shading of identical and similar residues was done using the following groups of amino acids: hydrophobic residues(GAVLMI), polar residues(STCPNQ), aromatic residues(FYW), acidic residues(DE), basic residues(KR), Histidine(H). The cladogram was generated using TreeView. All predictions of functional domain and secondary structure were done using the Pfam database of Hidden Markov Models and the Jpred secondary structure prediction, respectively. Sequence database searching was performed using BLAST on GenBank databases containing either ESTs or genomic sequence information.

### *Transient transfection of HEp-2 cells*

For transfection the hRrp4p, hRrp40p, hRrp41p and hRrp46p open reading frames were cloned into the pCI-neo vector (Promega), in frame with the vesicular stomatitis virus G epitope (VSV-G tag) to allow expression of N-terminally VSV-tagged proteins. For immunoprecipitation analyses approximately 10 x $10^6$ HEp-2 cells (grown to 80% confluent monolayers) were transfected with 30-40 µg of plasmid DNA in 1600 µl of DMEM containing 10% FCS by electroporation, which was performed at 260V and 950 µF using a Gene-Pulser II (Bio-Rad). After overnight culturing, cells were washed twice with PBS, resuspended in 500 µl of lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 1 mM DTE, 0.5 mM PMSF and 0.05% NP-40) and homogenized by sonication.

### *Immunoprecipitation*

Monoclonal mouse anti-VSV-G tag antibodies were coupled to protein G-agarose beads (Biozym) in IPP500 (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40) by incubation for 2 h at room temperature. Beads were washed twice with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40). For immunoprecipitations, cell extracts were incubated in IPP150 with the antibody-coated beads for 1 h at 4°C. Subsequently, beads were washed three times with IPP150 and precipitated proteins were eluted from the beads by the addition of SDS sample buffer. Finally, samples were resolved by 11.5% SDS-PAGE and were blotted on nitrocellulose membranes.

*Western blot analysis*

For western blot analysis, α-hRrp4p and α-hRrp41p rabbit antisera (14) were diluted 250-fold in blocking buffer (PBS containing 4% non-fat dried milk and 0.1% NP-40). As secondary antibody, horseradish peroxidase-conjugated swine anti-rabbit IgG (Dako Immunoglobulins) was used after 1000-fold dilution in blocking buffer. Visualization was performed by chemiluminescence.

# Chapter 4

## The origin of mitochondrial polynucleotide phosphorylase

Reinout Raijmakers
Léon Peeters
Wilma Vree Egberts
Walther J. van Venrooij
Ger J.M. Pruijn

*Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, Nijmegen, The Netherlands*

submitted for publication

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Polynucleotide phosphorylase (PNPase) plays a key role in the degradation of different RNA species in bacteria. The eukaryotic exosome complex is both structurally and functionally related to the bacterial PNPases. Recently, PNPase homologues have been identified in eukaryotes, the first of which was found to reside in spinach chloroplasts. Mammalian PNPases appeared to contain N-terminal extensions reminiscent of mitochondrial import signals and were proposed to be involved in the degradation of mitochondrial RNAs. Here, we demonstrate that the human PNPase indeed accumulates in mitochondria, which is mediated by an N-terminal transition peptide. In addition, we show that both RNase PH domains of human PNPase display exoribonuclease activity *in vitro*. Phylogenetic data indicate that the mitochondrial and chloroplast RNA degradation machinery has evolved from the eubacterial PNPase. In contrast, the eukaryotic proteins involved in nuclear and cytosolic RNA degradation and processing, i.e. the exoribonucleases associated with the exosome, are more closely related to archaeal proteins. Finally, we discuss the alternative exoribonucleases which are used by organisms lacking both PNPase and the exosome complex. Taken together, our data are fully consistent with a eubacterial origin of the organellar exoribonuclease machinery and an archaeal origin of the nuclear and cytosolic RNA degradation machinery.

## Introduction

The processing and degradation of RNA is a process equally important for the organisms of all three domains of life, eubacteria, archaebacteria and eukaryotes. Many (stable) RNAs present in the cell require extensive modification after their transcription to become functional. Three of the four eukaryotic ribosomal RNAs (rRNAs), for example, are transcribed as a single precursor molecule which subsequently undergoes a number of endoribonucleolytic cleavages, exoribonucleolytic processing at the RNA termini and single nucleotide modifications to obtain the mature rRNAs (101). Also, degradation of mRNA plays an important role in the regulation of gene expression, since removal of its mRNA will prevent the synthesis of new copies of a protein (102). To accomplish efficient processing and degradation of RNA, several molecular machines have evolved. Key enzymes in the degradation of prokaryotic RNA are exoribonucleases, which work in a 3' – 5' manner (103), whereas in eukaryotes RNA can be degraded both by 3' – 5' and by 5' – 3' exoribonucleases (56).

All known exoribonucleases can be classified in one of seven protein families. For some families (YhaM and RBN) members are only found in prokaryotes, whereas members of the 5PX exoribonuclease family (the only family of 5' – 3' exoribonucleases) are exclusively present in eukaryotes. Exoribonucleases belonging to the DEDD and RNR families are usually only found in eubacteria and eukaryotes and the proteins of the RRP4 family in archaebacteria and eukaryotes. The PDX family is represented in all three domains of life (104,105). The PDX proteins are characterized by the presence of one or two domains homologous to RNase PH (RPDs) and representatives of this family are associated with the eubacterial degradosome complex (PNPase) and the eukaryotic exosome

(Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, Mtr3) (10,106). In addition, the domains present in the RRP4 family members of the human exosome (Rrp4p, Rrp40p and Csl4p), the S1 and KH RNA binding domains, are also found in eubacterial PNPase. Recently, a structural model has been proposed for the eukaroytic exosome, partly based on the structural homology between exosome subunits and eubacterial PNPase (21,106). The exoribonuclease families and a few examples of proteins belonging to each family are listed in Table 1.

The proteins RNase II and RNase R (RNR family) and PNPase (PDX family) are the most important exonucleases in eubacteria and members of both families are present in virtually all eubacteria that have been sequenced to date. A double-minus mutant of RNase II and PNPase is lethal in *Escherichia coli* and, although some eubacteria are known that lack either RNR family exoribonucleases (e.g. the Actinobacteria) or PDX exoribonucleases (e.g. Mycoplasma) (103), only one eubacterium has been found lacking both enzymes (*Streptomyces coelicolor*). Remarkably, the domain of the archaebacteria has developed a different set of enzymes to deal with RNA degradation. In most archaea, no RNR family exoribonucleases and PNPase proteins are found. Instead, the genomes of these organisms usually encode two proteins that show homology to *E. coli* RNase PH (PDX family) and one protein homologous to the yeast exoribonuclease Rrp4p (RRP4 family) (104). Most likely, these archaeal proteins assemble into a single complex similar to the yeast and human exosome (86). Eubacteria usually also encode a single RNase PH homologue but in *E. coli* this gene (which is involved in tRNA maturation) is not essential, except in strains lacking a large number of other ribonucleases (107). Of all sequenced archaeal genomes, only two, those of *Methanococcus jannaschii* and *Halobacterium* NRC-1, do not contain genes encoding exosome subunits. To compensate for the missing exosome complex, both have acquired alternative enzymes to deal with RNA degradation. A homologue of the protein YhaM, which was recently identified as an exoribonuclease in *Bacillus subtilis* (105), is present in *M. jannaschii*,

**Table 1. Overview of exoribonuclease families**

| Family | Activity | Distribution | Prokaryotic examples | Eukaryotic examples |
|---|---|---|---|---|
| YhaM | 3'-5' | some eubacteria<br>some archaea | YhaM | - |
| RBN | 3'-5' | some eubacteria | RNase BN | - |
| 5PX | 5'-3' | all eukaryotes | - | Xrn1<br>Rat1 |
| DEDD | 3'-5' | some eubacteria<br>all eukaryotes | RNase D | PM/Scl-100 |
| RNR | 3'-5' | most eubacteria<br>all eukaryotes | RNase R<br>RNase II | Dis3<br>Msu1 |
| PDX | 3'-5' | all domains | PNPase (eubacteria)<br>RNase PH (eubacteria)<br>exosome subunits (archaea) | PNPase<br>exosome subunits |
| RRP4 | 3'-5' | most archaea<br>all eukaryotes | exosome subunits | exosome subunits |

**Table 2. The occurrence of functional exoribonuclease genes of the PDX, RNR and RRP4 families in the three domains of life.**

| | | | all required for a functional exosome | | |
|---|---|---|---|---|---|
| | PNPase proteins | RNase R/II proteins | RNase PH-like proteins | | RRP4-like proteins |
| Exoribonuclease family | PDX | RNR | PDX | PDX | RRP4 |
| COGs[1] | COG1185 | COG0557 | COG0689 | COG2123 | COG1097 |
| Eubacteria (general) | 1 | 1/2 | 1 | - | - |
| *B. subtilis* | 1 | 2 | 1 | - | - |
| Mycoplasma | - | 1 | - | - | - |
| Actinobacteria | 1 | - | 1 | - | - |
| *S. coelicolor* | - | - | 1 | - | - |
| Archaebacteria (general) | - | - | 1 | 1 | 1 |
| *Halobacterium* NRC-1 | - | 1 | - | - | - |
| *M. jannaschii* | - | - | - | - | - |
| Eukaryotes (general) | 0-2 | >2 | 3 | 3 | 3 |
| *S. cerevisiae* | - | >2 | 3 | 3 | 3 |
| *A. thaliana* | 2 | >2 | 3 | 3 | 3 |
| *H. sapiens* | 1 | >2 | 3 | 3 | 3 |

[1] The COG number corresponds to the entry number of a particular protein group in the clusters of orthologous groups (COGs) database.

whereas *Halobacterium* NRC-1 contains an RNR family exoribonuclease.

In eukaryotes, the situation is somewhat more complex, because there is 5'-3' RNA degradation in addition to 3'-5' RNA degradation. In *Saccharomyces cerevisiae* 5'-3' degradation is the main pathway for mRNA degradation, whereas in human cells the 3'-5' pathway is the more common mechanism. A major player in the latter pathway is the exosome (56), which mediates the degradation and processing of a large number of different RNA species in yeast and human cells (106). Homologues of exosome subunits are found in all eukaryotes, including plants (26). An overview of the number of functional exoribonuclease genes of the PDX, RNR and RRP4 families in eubacteria, archaebacteria and eukaryotes is given in Table 2.

The protein PNPase was long believed to be an exclusively eubacterial protein until it was also found in chloroplasts of *Spinacia oleracea* (90). Recently, we have shown the existence of nuclear genes encoding PNPase homologues in *Homo sapiens* and *Mus musculus* and confirmed that these genes are indeed expressed. Surprisingly, the polypeptides encoded by these genes both appeared to contain putative mitochondrial localization signals (106). A comparison of the schematic structures of the eubacterial, mammalian and spinach PNPases is shown in Figure 1.

All PNPases contain the main structural domains in the same order: two RPDs separated by an alpha-helical domain and KH and S1 domains near the C-terminus. The main differences are the length of the alpha-helical domain and the presence of mitochondrial and chloroplast transition peptides (TPs) near the N-termini of the eukaryotic PNPases. Remarkably, the spinach protein also contains a mitochondrial transition peptide (mTP) flanking the chloroplast transition peptide (cTP, Figure 1).

In this study we investigated the subcellular localization of the human PNPase, which indeed appeared to accumulate in the mitochondria. In addition, we present phylogenetic data indicating that the mitochondrial and chloroplast RNA degradation machinery has evolved from the eubacterial PNPase and that the exoribonucleases associated with the exosome are more closely related to archaeal proteins.

## Results and Discussion

### *Subcellular localization of human PNPase*

To investigate the subcellular localization of the mammalian PNPases, the human cDNA was cloned into the pEGFP-N3 vector, in-frame with the coding sequence of the enhanced green fluorescent protein (EGFP). As can be seen in Figure 2, analysis of HEp-2 cells expressing the PNPase-EGFP fusion protein by confocal microscopy indeed demonstrated a mitochondrial localization for the human PNPase protein (Figure 2, panels A-C). A detailed comparison of the staining by PNPase-EGFP and by MitoTracker-Red (Figure 2, panels D-F) suggests that the distribution of PNPase in the mitochondria is not uniform. For yet unknown reasons PNPase seems to be enriched in certain regions of the mitochondria. Transfection of HEp-2 cells with a construct containing the PNPase sequence at the C-terminus of EGFP (pEGFP-C3 vector) resulted in a diffuse cytoplasmic staining (Figure 2, panel G) (111). The latter result is most likely due to masking of the mitochondrial import signal at the N-terminus of PNPase in the EGFP-PNPase fusion protein. Note that mitochondrial transition peptides are often located close to the N-terminus of mitochondrial proteins (100). To confirm the presence of an N-terminal mTP in the human PNPase protein, the sequence encoding the first 45 amino acids of this protein was cloned into the EGFP-N2 vector, in-frame with the coding sequence of EGFP. The results in Figure 3 demonstrate that this N-terminal element, which shows structural characteristics similar to known

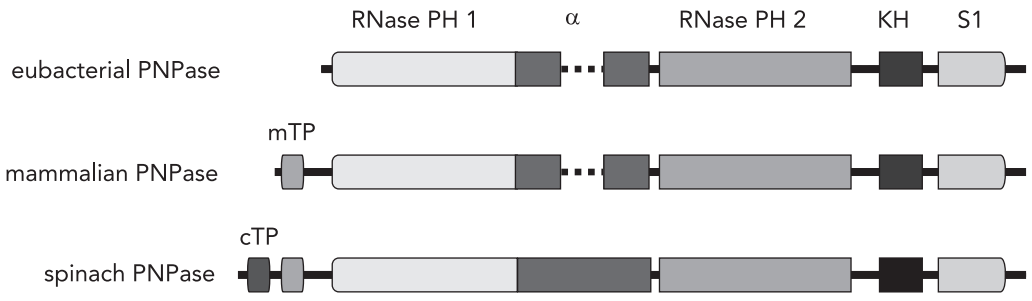


**Figure 1. Schematic representation of the structure of eubacterial and eukaryotic PNPases.**
The relative positions of the chloroplast and mitochondrial transition peptides (cTP and mTP), the two RPDs, the α-helical domain, and the KH and the S1 RNA binding domains are indicated.

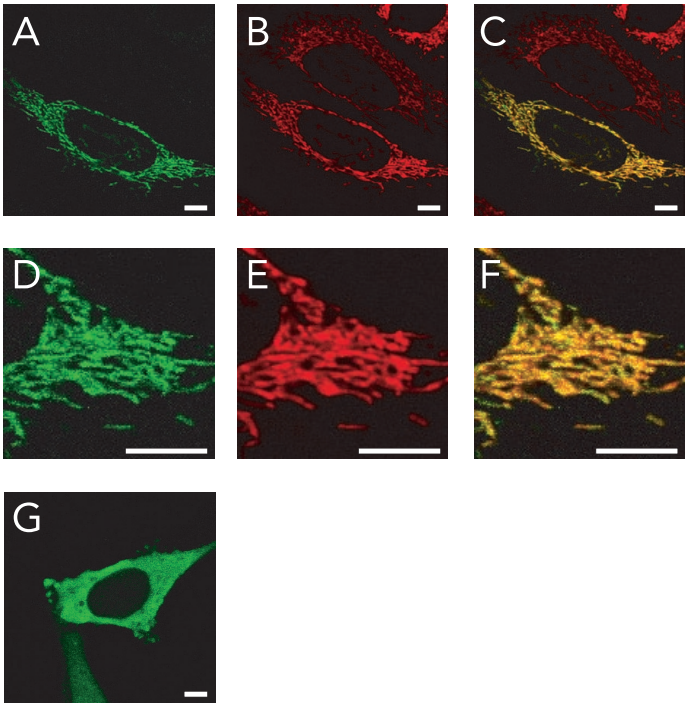


**Figure 2. Mitochondrial localization of human PNPase.**
HEp-2 cells were transfected with a construct encoding EGFP fused to the C-terminus of hPNPase. After 16 hours the expressed fusion proteins were visualized by confocal fluorescence microscopy (panel A). In panel B, the mitochondria of the cells were visualized using MitoTracker-Red CMX Ros. The overlay of both images is shown in panel C. Panels D-F show a magnification of a part of the lower cell shown in panels A-C. Panel G shows the diffuse cytoplasmic staining by a fusion protein of EGFP fused to the N-terminus of hPNPase. Each bar represents 5 μm.

mTPs (112), was indeed able to target EGFP to the mitochondria of HEp-2 cells.

Because mTP-like elements are also found in the PNPase homologues of other animals, we predict that this enzyme also resides in the mitochondria of other animals and possibly all other multicellular eukaryotes. In this respect, it is important to note that the only completely sequenced plant genome, that of *Arabidopsis thaliana*, encodes two distinct PNPases, one which is predicted to accumulate in chloroplasts and another which contains a putative mitochondrial localization signal (113). Apparently, eukaryotic PNPases are important for the degradation and/or processing of RNA in both main eukaryotic organelles of eubacterial origin (it is believed that eubacteria engulfed by primitive eukaryotes led to a symbiosis of the two organisms and that the engulfed bacteria later evolved into the mitochondria and chloroplasts (114)). The eubacteria

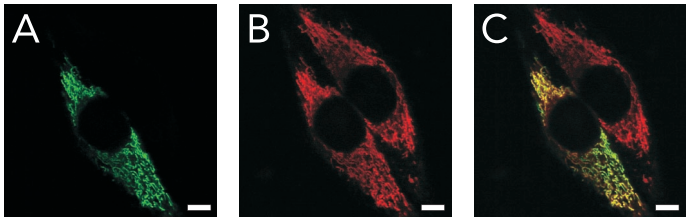


**Figure 3. Human PNPase contains an N-terminal mitochondrial import signal.**
HEp-2 cells were transfected with a construct encoding the first 45 amino acids of PNPase fused to the N-terminus of EGFP. After 16 hours the fusion protein was visualized by fluorescence microscopy (panel A). In panel B, the mitochondria of the same cells were visualized using MitoTracker-Red CMX Ros. Panel C shows the overlay of both images. Each bar represents 5 μm.

that are most closely related to the chloroplasts and the mitochondria (*Synechocystis* sp. and *Rickettsia prowazekii*, respectively (115)) both contain a PNPase encoding gene. A comparison of the amino acid sequences of a number of eubacterial and eukaryotic PNPases and of their exosome counterparts in human and archaea demonstrated that all known mitochondrial PNPase sequences from animals are more closely related to each other than to those of the eubacterial or chloroplast PNPases (Figure 4). To obtain a reliable alignment of exosome subunits and PNPase proteins, the Pfam database (96) was used to determine the borders of the RPDs and S1 RNA binding domains in these proteins. Subsequently, the amino acids sequences of the individual domains were combined to obtain pseudoproteins encompassing the RPDs and the S1 domains of exosome and PNPase proteins from a number of organisms. For the human and archaeal exosome, the domains were combined according to our current knowledge on the assembly of exosome proteins in a PNPase-like structure (106). These pseudoproteins were used to generate an alignment and a phylogram demonstrating the relationship between the proteins. The *A. thaliana* mitochondrial PNPase can not be clearly grouped with either the chloroplast or the mitochondrial PNPases, but does not contain a chloroplast transition peptide (cTP). Future studies and the completion of other plant genomes will learn whether all plants, like *A. thaliana*, contain two distinct PNPases. It would not be surprising if some plants contain only a single functional PNPase gene that might suffice

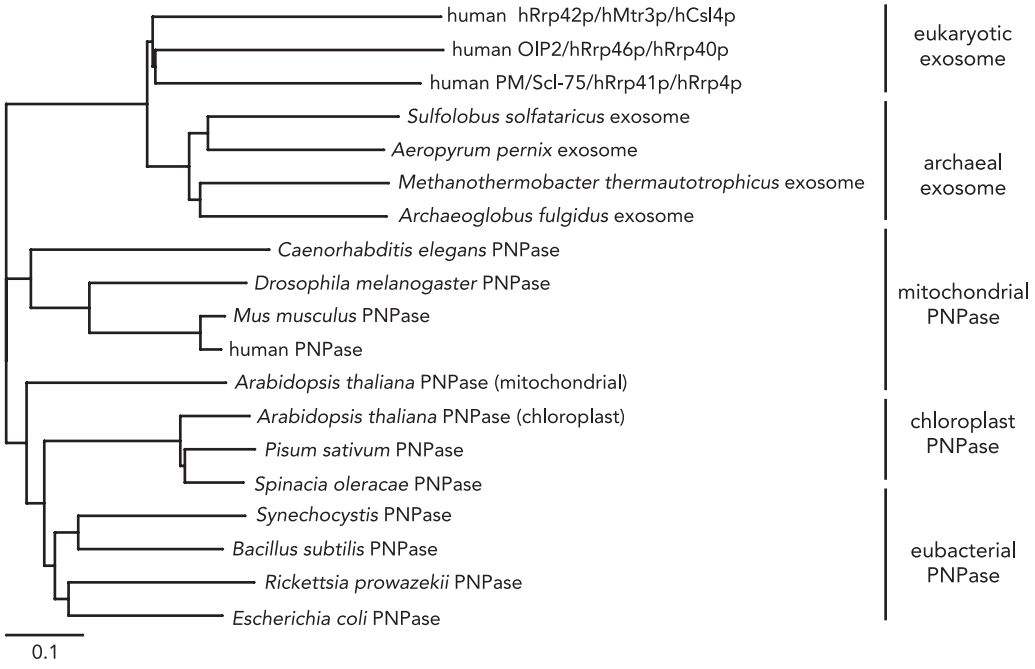


**Figure 4. Phylogram of PNPase and exosome proteins of several prokaryotic and eukaryotic organisms.** The phylogram was generated based upon a ClustalW multiple sequence alignment of pseudoproteins composed of the two RPDs and the S1 domain of exosome subunits or of the PNPase. The human exosome subunits were combined based upon structural models for this complex: hRrp42p/hMtr3p/hCsl4p, OIP2/hRrp46p/hRrp40p, and PM/Scl-75/hRrp41p/hRrp4p. The scale bar corresponds to 0.1 substitutions per site.

for both the chloroplasts and the mitochondria, since a number of other plant proteins have been identified that can enter both organelles (115). Moreover, all identified chloroplast PNPase proteins contain a putative mTP in addition to the cTP (see schematic structure for spinach PNPase in Figure 1). The homology between eubacterial and nuclear encoded organellar proteins is not only observed for PNPase, but also for other proteins, for example some proteins involved in RNA metabolism, like the organellar RNA polymerases. The RNA polymerases of eubacteria are more homologous to the chloroplast and mitochondrial RNA polymerases of eukaryotes than to the nuclear RNA polymerases, which display more homology to archaebacterial RNA polymerases (data not shown). Similar relationships between nuclear and archaeal sequences and between organellar and eubacterial sequences have been observed for the ribosomal protein rpl14 (116).

*Evolution of PNPase*

Since there is significant homology in domains and structure between PNPase and exosome proteins it is not unlikely that these protein have a common ancestor. In Figure 5 a model for the evolution of these proteins is presented. The earliest common ancestor of PNPase and the exosome most likely has been a protein containing a single RPD. Domain duplication of the RPD, followed by the addition of the KH and S1 domains might have resulted in a polypeptide displaying the same modular structure as present-day PNPases (although the KH and S1 domains might have been attached already to the protein containing only one RPD). Shortly after RPD duplication, both copies of this domain have diverged with conservation of the exoribonuclease activity in the second domain (RPD2). The N-terminal domains (RPD1) display less homology to the PDX family than RPD2 and the ancient RPD1 has therefore been suggested to have lost its exoribonuclease activity and may have acquired a new (enzymatic) activity (94). In eubacteria PNPases are still present, although these organisms often contain an additional RNase PH protein, which most likely originated from a duplication of the RPD2 of eubacterial PNPase. In archaea and eukaryotes, the domains of the ancient PNPase protein were separated in three individual proteins. Interestingly, the three exosome genes display a tandem arrangement in the genomes of many archaebacteria (86). Remarkably, in comparison with all PNPases the relative positioning of the S1 and KH domains has switched in all 'descendants', both archaeal and eukaryotic (referred to as the RRP4 exoribonuclease family). In eukaryotes, a number of gene duplications led to the presence of six RPD containing exosome subunits and three RRP4-type exosome proteins (of which one has lost the KH domain). The hypothesis that eubacterial RNase PH originates from a duplication of RPD2 (and thus is exclusively present in eubacteria), and thus is only distantly related to the RPD-type exosome subunits, is supported by the observation that the RPD of eubacterial RNase PH has a higher degree of homology with the RPD2 of eubacterial PNPase than with eukaryotic exosome subunits (data not shown). Finally, PNPase was introduced into eukaryotes concomitant with the generation of mitochondria and chloroplasts. Later, a number of eukaryotes may have lost the PNPase gene during evolution.

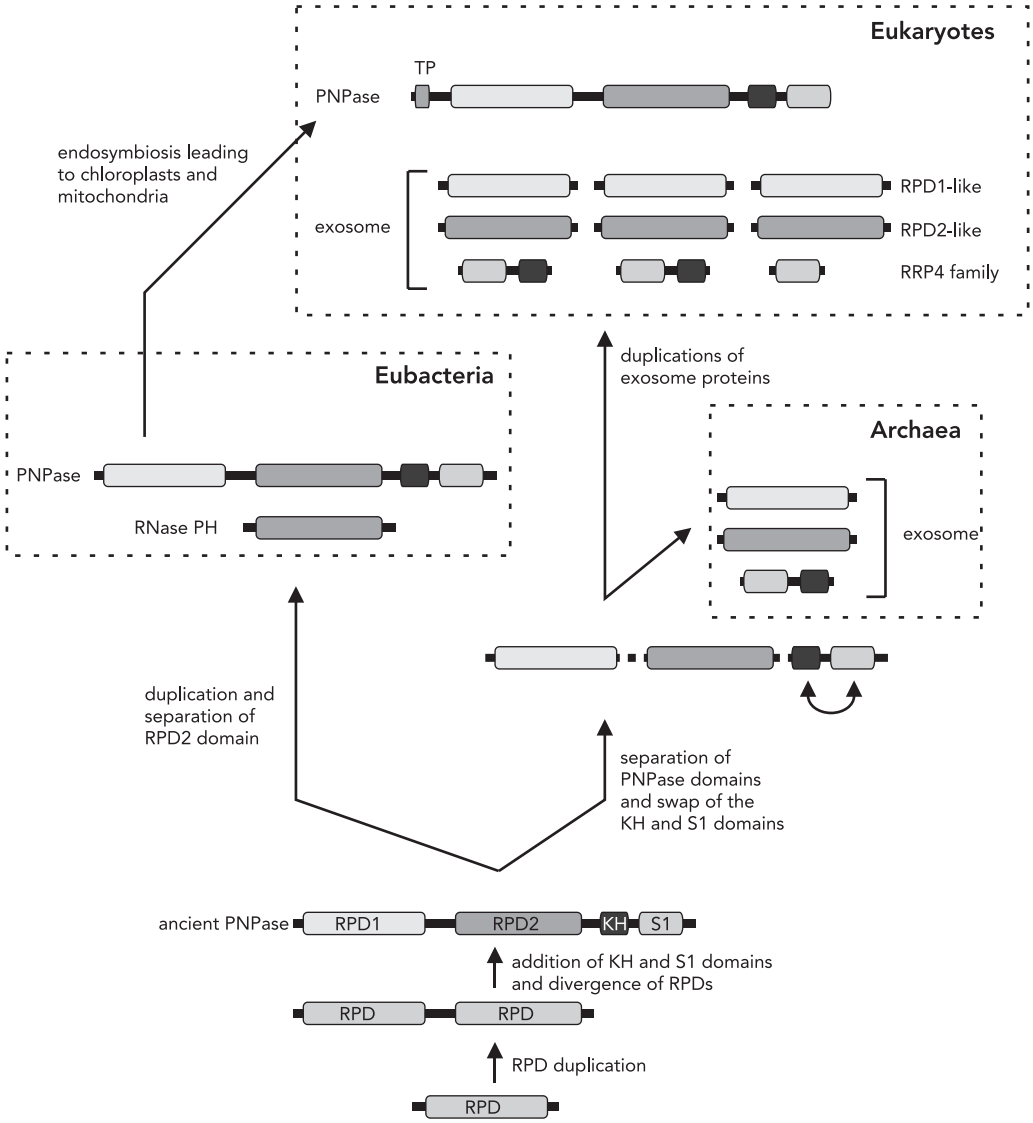


**Figure 5. Model for the evolution of eukaryotic PNPase and the exosome.**
A schematic overview of the evolutionary events leading from one common ancestor to the presence of both PNPase and the exosome in present day multicellular eukaryotes. Note that the α-helical domain of PNPase has been omitted from this model.

*Exoribonuclease activity of human PNPase*

The divergence between the sequences of the two RPDs in PNPase has been suggested to reflect a functional difference, in particular because only RPD2 appeared to contain an active site similar to that of eubacterial RNase PH (94). To analyse which domains of the human PNPase contains ribonuclease activity, constructs were generated encoding either RPD1, RPD2 or the combined KH and S1 domain, each fused to the C-terminus of GST. After expression and purification the ribonuclease activity of the fu-

sion proteins was analysed by incubation at 37°C with a uniformly $^{32}$P-labeled substrate RNA. As can been seen in Figure 6, both RPDs exhibited ribonuclease activity (panel A, lanes 5-10), in contrast to the KH/S1 domain (lanes 11-13) and GST alone (lanes 2-4). Remarkably, both RPDs appeared to degrade the substrate RNA in a different manner. The results are consistent with a processive exoribonuclease activity for RPD1 and a distributive exoribonuclease activity for RPD2. Moreover, the activity of RPD1 appeared to be independent of phosphate, whereas that of RPD2 requires phosphate (results not shown). Previously, the RPD1 domain has been suggested to be functionally distinct from RPD2 and RNase PH, since PNPase of *S. antibioticus*, which was crystallized in the presence of the phosphate analog tungstate, only showed incorporation of tungstate in RPD2 and not in RPD1 (94). Thus surprisingly, although being evolutionarily related to RNase PH, the RPD1 of PNPase seems to have lost its dependency for phosphate to degrade RNA.

In agreement with recently published data (111), also the full-length human PNPase displayed exoribonuclease activity *in vitro*, as demonstrated by the degradation of the substrate RNA by the PNPase-EGFP fusion protein immunoaffinity-purified from transfected HEp-2 cell extracts. Degradation by the full-length PNPase appeared to proceed mainly in a processive manner (Figure 6, panel B). In bacteria, PNPases generally display phosphate dependent processive activities and RNase PH distributive activities (104).

With regard to the function of human PNPase in mitochondria, it has been described that stimulation of human cells with interferons leads to upregulation of PNPase (111) and a decrease in the levels of most of the 13 mitochondrial mRNAs (117), strongly suggesting a role for PNPase in the regulation of mitochondrial mRNA levels.

### *PNPase deficient eukaryotes*

Although PNPase genes seem to be present in all multicellular eukaryotes (106), no genes en-

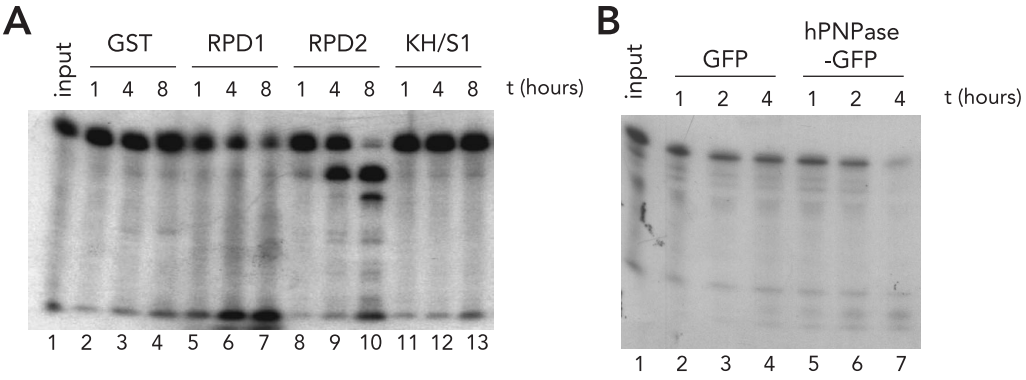


**Figure 6. Exoribonuclease activity of human PNPase *in vitro*.**
Recombinant GST fusion protein of several PNPase domains (panel A) or material (co-)precipitated with anti-EGFP antibodies from HEp-2 cells expressing hPNPase-EGFP (panel B) was incubated with a uniformly $^{32}$P-labeled substrate RNA of 37 nucleotides. Samples were taken at the indicated time points and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Either GST alone (panel A) or GFP alone (panel B) was used as a negative control.

coding PNPase homologues exist in the genomes of *S. cerevisiae* or *Schizosaccharomyces pombe*.

The mitochondria of *S. cerevisiae*, however, are known to contain a different enzyme-complex involved in the degradation of RNA, the mitochondrial degradosome (also designated mtEXO complex). This complex contains an exoribonuclease belonging to the RNR family (MSU1) in addition to an RNA helicase (118). Apparently, in comparison with multicellular eukaryotes, which do not encode an obvious homologue of MSU1, the mitochondria of yeast have developed an alternative machinery for the degradation of RNA. Interestingly, the organism which is believed to be most closely related to eukaryotic mitochondria, *R. prowazekii*, does not contain genes encoding RNR family exoribonucleases, but, as described above, does contain a PNPase gene. The apparent difference in the mitochondrial RNA degradation machinery between yeasts and multicellular eukaryotes is difficult to explain. A possibility is that the eubacterial ancestor of mitochondria contained exoribonucleases of both the PDX and RNR families and that eukaryotes have lost one of these, PDX in yeasts and RNR in other eukaryotes. Similarly, *R. prowazekii* may have lost the RNR-type exoribonuclease. Another possibility is that the RNR-type gene in yeasts is of nuclear instead of mitochondrial origin.

The eukaryotic PDX- and RNR-type exoribonucleases are all encoded by nuclear genes. This is probably the result of the transfer of genes from the mitochondrial to the nuclear genome by horizontal gene transfer, which is very common for genes encoding mitochondrial proteins.

## Concluding remarks

The observation that all organisms with completely sequenced genomes (except for *M. jannaschii*) appear to encode RNA degrading enzymes similar to PNPase and/or RNase II / RNase R underscores the essential role of these enzymes for life on earth. Especially the PDX family-based RNA degradation machinery (in the form of either PNPase or the exosome) is widely spread throughout all three domains of life, eubacteria, archaebacteria and eukaryotes.

The presence of these proteins in eukaryotic organelles is most likely required for the degradation and processing of RNAs encoded by the organellar genomes. Very recently it has been reported that overexpression of human PNPase in melanoma cells leads to cell-cycle arrest and apoptosis, following down-regulation of the nuclear-encoded gene c-myc, similar to the effects of retinoic acid-induced cell cycle arrest (119). Since retinoic acids are known to lead to mitochondrial instability prior to apoptosis (120), the overexpression of human PNPase might lead to similar mitochondrial defects, followed by apoptosis.

Although our current knowledge on eukaryotic PNPases is consistent with the theory that mitochondria and chloroplasts have evolved from symbiotic eubacteria, it remains unclear why some eukaryotes have developed a completely different machinery for mitochondrial RNA degradation (PNPase based in multicellular eukaryotes, RNase R based in yeasts).

## Material and Methods

*Transient transfection of HEp-2 cells and fluorescence microscopy*

All transfections were carried out as described before (106). Briefly, the open reading frame of human PNPase was cloned into the pEGFP-N3 vector (Clontech), in frame with the enhanced green fluorescent protein (EGFP). Approximately 2 x $10^6$ cells were transfected with 10-20 μg of DNA in 800 μl of DMEM containing 10% FCS by electroporation. After transfection, cells were seeded onto coverslips and cultured overnight. Cells were washed twice with PBS and mounted in colourless medium. To allow visualization of mitochondria, cells were incubated with MitoTracker-Red CMX Ros (Molecular Probes), diluted 1:25,000 in medium for 3 minutes prior to analysis. Confocal fluorescence microscopy was performed using a Leica DM IRBE confocal microscope with living cells.

*Expression and purification of recombinant proteins*

The GST (fusion) proteins were expressed in bacteria and purified as described previously (87). After expression and affinity-purification by gluthatione-Sepharose chromatography, recombinant proteins were further purified by ammoniumsulfate precipitation to remove all traces of bacterial ribonuclease activity. Finally, the proteins were dissolved in PBS.

*Immunoprecipitation*

Polyclonal rabbit antibodies were coupled to Protein A-agarose beads (Biozym) in IPP500 (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40) by incubation for 2 h at room temperature. Beads were washed twice with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40). For each immunoprecipitation, cell extract was incubated with the antibody-coupled beads for 1 h at 4°C. Subsequently, beads were washed three times with IPP150 and two times with buffer A (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM $Na_2HPO_4$).

*Exoribonuclease activity assay*

Substrate solution (approx. 100 ng $^{32}$P-labeled substrate in 40 μl buffer A) was added to the immunoprecipitates or to approximately 10 μg of purified recombinant protein and the samples were incubated at 37 °C with gentle agitation. Formamide loading buffer was added to 10 μl samples taken at regular intervals and these were immediately frozen. Samples were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. Substrate RNA was generated by in vitro transcription in the presence of [$^{32}$P]UTP, using T3 RNA polymerase and an XbaI-linearized pBS(-) (Stratagene) plasmid as template.

*Bioinformatics*

Multiple alignments of PNPase and exosome amino acid sequences were generated with ClustalW, using BLOSUM30 substitution matrices. To obtain a reliable alignment of exosome subunits and PNPase proteins, the exact position of the RNase PH and S1 domains in these proteins was determined using Pfam (96). The amino acid sequences of these domains were subsequently combined into a single pseudoprotein, which was used in the alignment. The phylogram was generated using TreeView. The presence of specific genes in the fully sequenced genomes of prokaryotes was analysed using the database of COGs (Clusters of Orthologous Groups) (108) and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) at the EMBL (109). Secondary structure predictions were performed using the PSIPRED algorithm (110).

# Chapter 5

## The association of the human PM/Scl-75 autoantigen with the exosome is dependent on a newly identified N-terminus

Reinout Raijmakers
Wilma Vree Egberts
Walther J. van Venrooij
Ger J.M. Pruijn

*Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, Nijmegen, The Netherlands*

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*The exosome is a complex of 3′ → 5′ exoribonucleases, which functions in a variety of cellular processes, all concerning the processing or degradation of RNA. Paradoxically, the previously described cDNA for the human autoantigenic exosome subunit PM/Scl-75 (Alderuccio et al. (1991) J.Exp.Med. 173, 941-952) encodes a polypeptide that failed to interact with the exosome complex. Here, we describe the cloning of a more complete cDNA for PM/Scl-75 encoding 84 additional amino acids at its N-terminus. We show that only the longer polypeptide is able to associate with the exosome complex. This interaction is most likely mediated by protein-protein interactions with two other exosome subunits, hRrp46p and hRrp41p, one of which was confirmed in a mammalian 2-hybrid system. In addition we show that the putative nuclear localization signal present in the C-terminal region of PM/Scl-75 is sufficient, though not essential for nuclear localization of the protein. Moreover, the deletion of this element abrogated the nucleolar accumulation of PM/Scl-75, although its association with the exosome was not disturbed. This suggests that this basic element of PM/Scl-75 plays a role in targeting the exosome to the nucleolus.*

## Introduction

The PM/Scl-75 protein (encoded by gene PMSCL1) was the first protein of the PM/Scl complex to be characterized (8). The PM/Scl complex was originally described as a complex consisting of 11-16 proteins, some of which are autoantigenic in autoimmune patients. Autoantibodies to this complex are most frequently found in patients suffering from polymyositis/scleroderma (PM/Scl) overlap syndrome, but are also present in some patients with myositis or scleroderma alone (5,6). The subunits PM/Scl-75, PM/Scl-100 and hRrp4p have been shown to carry the main autoantigenic determinants for polymyositis/scleroderma overlap syndrome autoantibodies (78,79). Following the identification of two of its subunits, PM/Scl-75 and PM/Scl-100 (7,9) and their characterization as putative exoribonucleases (10) the PM/Scl complex was shown to be related to the yeast exosome, a complex containing approximately 10 exoribonucleases (11).

The exosome functions in a variety of processes involving the 3'→5' processing or degradation of RNA. Among these processes are the maturation of 5.8S rRNA (12,31,32), the processing of many small nuclear and nucleolar RNAs (33,38,42) and the turnover of different types of mRNAs (82,83), especially ARE (AU-rich element) containing mRNAs (17,57). PM/Scl-75 has been suggested to be an AU-rich element binding protein (AUBP), involved in the recruitment of the exosome to this class of mRNAs (57).

Like five other exosome proteins, PM/Scl-75 contains an RNase PH domain, which is homologous to the prokaryotic 3'-5' exoribonuclease RNase PH. Based on mutual interactions between exosome components and structural similarity with the bacterial protein polynucleotide phosphorylase (PNPase), a component of the bacterial degradosome, recently a model for the structure of the human exosome was generated. In this model the six proteins containing an RNase PH domain (RPD) form the

core of the exosome, which adopts a hexameric ring structure (106).

Cloning of a cDNA encoding the human PM/Scl-75 protein revealed an acidic polypeptide sequence of only 39.2 kDa. Its migration at 75 kDa in SDS-PAGE was explained by its highly charged C-terminal half (8). In immunofluorescence experiments the protein showed mainly nucleolar localization (8), although it was also found to be present in the cytoplasm and nucleoplasm of human cells (57). The N-terminal half of the protein shows homology to the bacterial RNase PH protein (10) and the C-terminal half is characterized by a region of approximately 100 amino acids highly enriched in acidic residues. Close to the C-terminus a putative nuclear localization signal (NLS) is located consisting of seven basic residues (KRRKKKR) (8). This cDNA of PM/Scl-75 has been used in a number of transfection experiments to study its subcellular localization and association with the exosome. Although similar experiments with most other exosome components were successful, EGFP fusion proteins of PM/Scl-75 were unable to enter the nucleolus and PM/Scl-75 failed to interact with other exosome components in a mammalian 2-hybrid system (106).

Here, we show that the previously described protein sequences of PM/Scl-75 are most likely incomplete. We have cloned and characterized additional cDNAs encoding PM/Scl-75 that in comparison with the original sequence, encode 84 additional amino acid residues at its N-terminus. In contrast to the previously described PM/Scl-75 polypeptide, this longer variant appeared to be able to associate with the exosome and to accumulate in the nucleolus.

## Results

### *Amino acid sequence of PM/Scl-75*

Several amino acid sequences for the human PM/Scl-75 polypeptide have been reported previously. Besides the first sequence ever described (here referred to as PM/Scl-75a-α, acc. number M58460) (8) a splicing variant containing the sequence encoded by an additional exon ('PM/Scl-75a-β', acc. number HSU09215) and a sequence containing 68 additional amino acid residues at the N-terminus ('PM/Scl-75b-α', acc. number Q06265) have been reported. Moreover, the sequence reported for *Mus musculus* PM/Scl-75 (acc. number Q9JHI7) contains, compared to the human PM/Scl-75a-α, 84 additional amino acids residues at the N-terminus (122). The reported heterogeneity in sequences for the N-terminal region of the human PM/Scl-75 prompted us to screen the human EST (expressed sequence tag) databases with the available human and mouse PM/Scl-75 cDNA sequences. These analyses demonstrated that in humans PM/Scl-75 sequences are expressed that fully correspond to the N-terminal region of the mouse protein. Thus, in comparison with PM/Scl-75a also the human protein may contain 84 additional amino acids. Based upon these data extended open reading frames for the human PM/Scl-75 were generated ('PM/Scl-75c-α' and 'PM/Scl-75c-β') and submitted to the EMBL database under accession numbers AJ505989 and AJ517294. Figure 1A shows the sequence of PM/Scl-75c in which the differences with previously described PM/Scl-75 sequences are indicated. In Figure 1B a schematic overview of the human polypeptides and their mRNAs is shown. It is important to note that PM/Scl-75a lacks a significant part of the RNase PH domain (RPD) as predicted by Pfam (96).

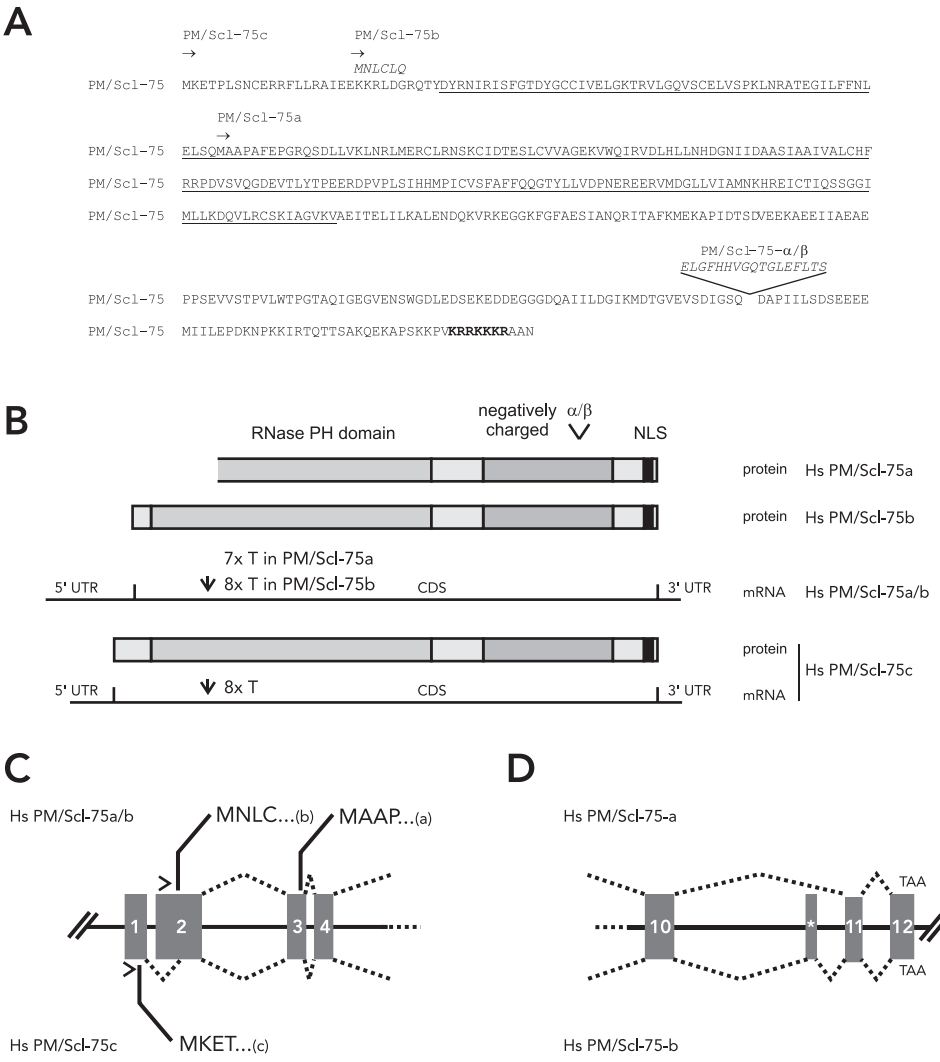


**Figure 1. The different isoforms of PM/Scl-75.**
A. Overview of the sequence of PM/Scl-75, in which the N-termini of the different isoforms are indicated (arrows and sequence in italics), as well as the position of the sequence encoded by the extra exon 10⋆ (sequence in italics indicated by PM/Scl-75-α/β). The RNase PH domain (underlined) and the putative NLS sequence (in bold) are also marked in the sequence. B. Schematic representation of the domains present in the different isoforms of the PM/Scl-75 protein. The RNase PH domain (which is incomplete in PM/Scl-75a), the negatively charged region, the site at which the amino acids encoded by the extra exon 10⋆ are inserted in PM/Scl-75-β and the putative nuclear localization signal (NLS) are indicated. Below the proteins schematic representations of the corresponding mRNAs are shown. The relative lengths of the coding sequence (CDS), the 5' and 3' untranslated regions (UTR) and the number of thymidine residues in the thymidine stretch are indicated. C. A schematic overview of the first 4 exons (1-4) of the PMSCL1 gene, its putative transcription initiation sites (marked '>') and mode of splicing, leading to the various N-terminally different forms of PM/Scl-75. The first four amino acids of these ORFs (a, b, and c) are indicated. D. A schematic overview of the last 4 exons (10, 10⋆, 11 and 12) of the PMSCL1 gene and its mode of splicing, leading to the α and β isoforms of PM/Scl-75. The exon present only in the β form of PM/Scl-75 is designated exon 10⋆ ('⋆' in the figure). The stop codon (TAA) is located in exon 12.

The RPD is expected to be important for the interactions of PM/Scl-75 with other exosomal proteins in the core of the exosome (106).

When the cDNA sequences are compared with the human genome, it is clear that the difference between the sequences is due to the use of an apparent alternative promotor in the PM/Scl-75 gene or to alternative splicing of the PM/Scl-75 pre-mRNA, as is illustrated in Figure 1C. The 5'-end of the cDNAs of PM/Scl-75c is encoded by an alternative first exon and lacks the first 234 nucleotides of the first exon of PM/Scl-75a. The protein sequence of PM/Scl-75b was derived from a cDNA very similar to that of PM/Scl-75a. The PM/Scl-75b cDNA contains a stretch of 8 instead of 7 thymidine residues at position 395-402 (which may be due to a sequencing error of the original clone), leading to a longer open reading frame (122). To investigate which of the corresponding mRNAs are actually expressed in human cell lines, RNA isolated from HEp-2, HeLa, 293, Jurkat and MOLT-4 cells was analysed by RT-PCR using primers specific for the PM/Scl-75a/b and PM/Scl-75c mRNAs. Both mRNAs were found to be present in all cells lines (results not shown). To discriminate between PM/Scl-75a and PM/Scl-75b, the RT-PCR products were cloned and sequenced. The results showed that the T-stretch consisted of 8 thymidine residues in all cases, indicating that the expressed mRNA corresponds to PM/Scl-75b. Strikingly, however, no evidence for the existence of mRNAs encoding either PM/Scl-75a or PM/Scl-75b was found in the EST databases, strongly suggesting that PM/Scl-75c is the most abundantly expressed isoform of the protein. To generate cDNAs encoding PM/Scl-75c, we performed PCR on human teratocarcinoma and placenta cDNA libraries using primers specific for the 5' region of PM/Scl-75c. The PCR products obtained were sequenced and found to be identical to the sequence of the cDNA encoding PM/Scl-75c as derived from the EST databases.

The polypeptides encoded by the PM/Scl-75a-α and PM/Scl-75c-α cDNAs were produced by in vitro transcription/translation and their migration in SDS-PAGE gels was compared with that of PM/Scl-75 from a cytoplasmic HeLa cell extract, which was detected by immunoblotting using anti-PM/Scl-75 rabbit antibodies (Figure 2). The results showed that the migration of

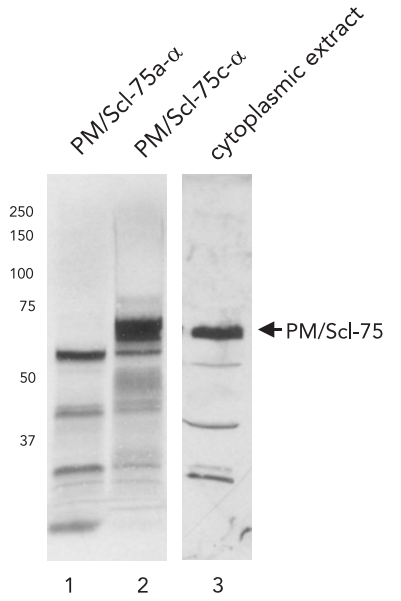


**Figure 2. The electrophoretic mobility of in vitro translated PM/Scl-75c-α is similar to that of HeLa cell PM/Scl-75.**
In vitro translated, $^{35}$S-labelled PM/Scl-75a-α (lane 1) and PM/Scl-75c-α (lane 2) were separated by SDS-PAGE in parallel with a cytoplasmic HeLa cell extract (lane 3) and transferred to a nitrocellulose blot. In vitro translated proteins were visualized by autoradiography and the lane containing the HeLa cell proteins was stained by immunoblotting using rabbit anti-PM/Scl-75 antibodies (note that PM/Scl-75-β is underrepresented in cytoplasmic HeLa extracts, as demonstrated in Figure 6). The position of molecular weight markers in the gel is indicated on the left.

PM/Scl-75c was identical to that of HeLa cell PM/Scl-75. The migration of in vitro translated PM/Scl-75a was clearly different, in agreement with previous observations (8).

To investigate the occurrence of the extra exon ('10⋆') that is present in the β variant of PM/Scl-75 (Figure 1D, exon a), the Genbank EST database was screened for sequences containing the exon-exon junctions 10⋆-11 and 10-11. In total, 52 ESTs encompassing this region were identified, 10 of which contained exon 10⋆, indicating that both splice variants of PM/Scl-75 are expressed, but that the isoform lacking exon 10⋆ may be more abundant.

Taken together, these results indicate that PM/Scl-75c-α is the predominant isoform of PM/Scl-75, but that at least four splice variants exist, which differ by their N-terminus and the presence or absence of 17 amino acids encoded by exon 10⋆.

### *The subcellular localization of PM/Scl-75*

To investigate the subcellular localization of PM/Scl-75 polypeptides, constructs encoding

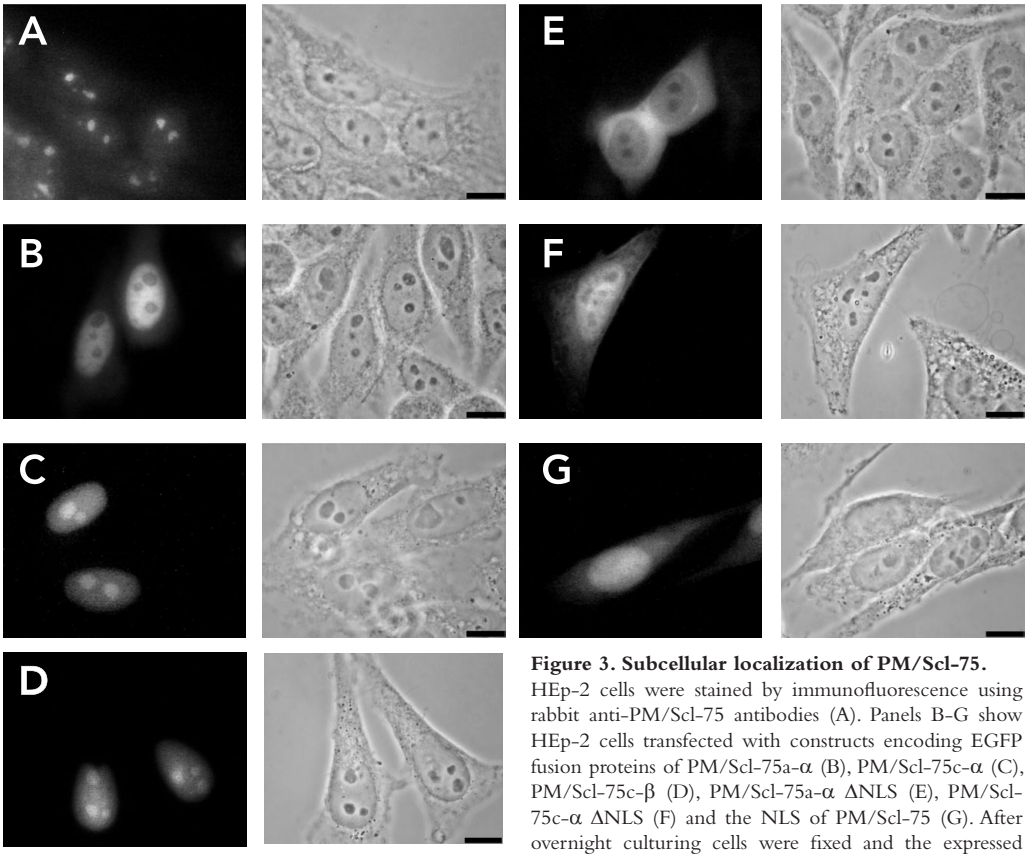


**Figure 3. Subcellular localization of PM/Scl-75.** HEp-2 cells were stained by immunofluorescence using rabbit anti-PM/Scl-75 antibodies (A). Panels B-G show HEp-2 cells transfected with constructs encoding EGFP fusion proteins of PM/Scl-75a-α (B), PM/Scl-75c-α (C), PM/Scl-75c-β (D), PM/Scl-75a-α ΔNLS (E), PM/Scl-75c-α ΔNLS (F) and the NLS of PM/Scl-75 (G). After overnight culturing cells were fixed and the expressed fusion proteins were visualized by fluorescence microscopy. The corresponding phase-contrast images are shown on the right of each fluorescence image. Each bar represents 10 μm.

PM/Scl-75a-α, PM/Scl-75c-α and PM/Scl-75c-β tagged with EGFP (enhanced green fluorescent protein) were generated. The fusion proteins were expressed in transiently transfected HEp-2 cells and as a control the endogenous PM/Scl-75 protein in HEp-2 cells was visualized by immunofluorescence using rabbit anti-PM/Scl-75 antibodies. Whereas the highest concentration of the endogenous PM/Scl-75 protein was found in the nucleoli (Figure 3, panel A), EGFP-PM/Scl-75a-α failed to enter the nucleoli and accumulated in the nucleoplasm (Figure 3, panel B). In contrast, EGFP-tagged PM/Scl-75c-α and PM/Scl-75c-β efficiently entered the nucleolus of HEp-2 cells (Figure 3, panels C and D. These data indicate that the N-terminal 84 amino acids of PM-Scl-75c are important for nucleolar accumulation and that the 17 amino acids encoded by exon 10* do not affect this process. To investigate whether the N-terminal region of PM/Scl-75c is sufficient for nucleolar targeting, the N-terminal 98 amino acids of PM/Scl-75c were fused to the N-terminus of EGFP and the subcellular localization of this fusion protein was analyzed in transfected HEp-2 cells. This fusion protein distributed throughout HEp-2 cells, similar to EGFP alone (data not shown). Previously, a sequence element (KRRKKKR) with similarity to nuclear

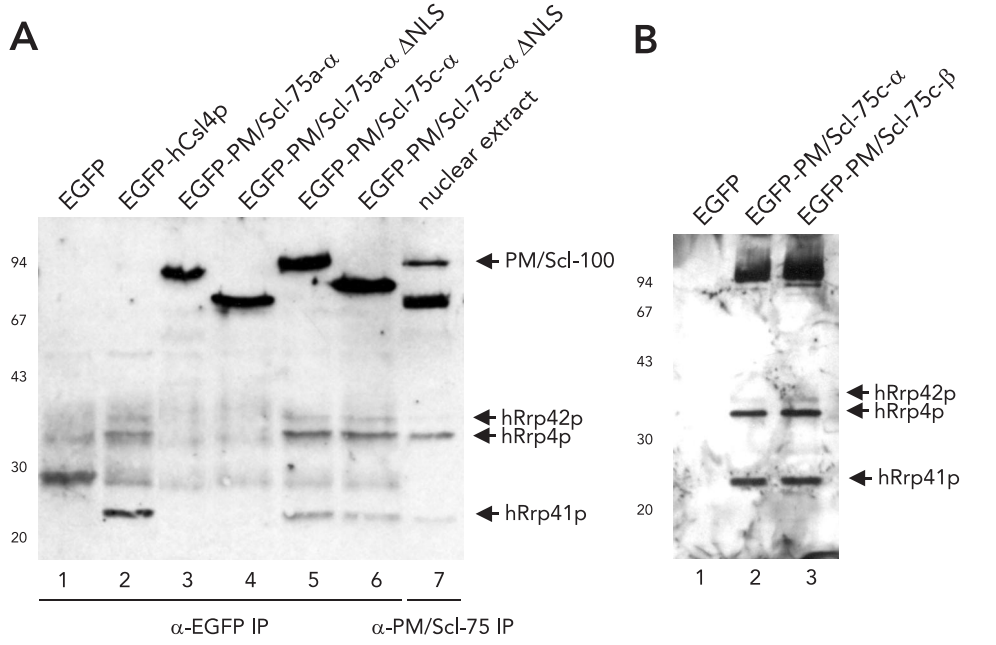


**Figure 4. Association of PM/Scl-75 with the human exosome.**

A. Co-immunoprecipitation experiments were performed using anti-EGFP antibodies and extracts of transfected HEp-2 cells expressing either EGFP alone (lane 1) or EGFP-fusion proteins of hCsl4p, PM/Scl-75a-α, PM/Scl-75a-α ΔNLS, PM/Scl-75c-α or PM/Scl-75c-α ΔNLS (lanes 2-6). Lane 7 contains proteins immunoprecipitated by anti-PM/Scl-75 rabbit antibodies from a nuclear HeLa cell extract. (Co-immunoprecipitated) proteins were visualized by immunoblotting using an anti-PM/Scl positive patient serum (Ven96). Arrows indicate the positions of co-precipitated autoantigenic exosomal proteins. The positions of molecular weight markers are indicated on the left.

B. A similar experiment as described above was performed using transfected HEp-2 cells expressing either EGFP alone (lane 1), PM/Scl-75c-α (lane 2) or PM/Scl-75c-β (lane 3).

localization signals (NLSs) has been reported to reside at the C-terminal end of PM/Scl-75 (8). To investigate the role of this element in nuclear and nucleolar entry, mutants of PM/Scl-75a-α and PM/Scl-75c-α lacking the C-terminal 39 amino acids were generated (ΔNLS). As can be seen in Figure 2E, PM/Scl-75a-α ΔNLS failed to enter the nucleus. Remarkably, PM/Scl-75c-α ΔNLS still was transported to the nucleus, but was unable to enter the nucleolus (Figure 2F). Finally, an EGFP fusion protein was expressed containing only the C-terminal 39 amino acids of PM/Scl-75. Figure 2G shows that this element is sufficient for transportation of EGFP to the nucleus. These data show that both N- and C-terminal elements of PM/Scl-75 are involved in its nuclear entry and that the C-terminal elements (also) play a role in nucleolar targeting.

*The association of PM/Scl-75 with the exosome*

In addition to elements directly involved in subcellular transport processes, also the association with the exosome may play a role in the subcellular localization. To investigate the effect of the mutations on the association with the exosome, immunoprecipitations with anti-EGFP antibodies were performed using lysates of HEp-2 cells transfected with constructs encoding the EGFP-tagged PM/Scl-75 mutants. The immunoprecipitated material was analyzed by western blotting, using a human serum (Ven96) reactive with several exosome proteins (including PM/Scl-100, PM/Scl-75, hRrp4p, hRrp41p and hRrp42p). The patient serum stained the different PM/Scl-75 fusion proteins very efficiently, which shows that all variants/mutants were expressed at similar levels. The results showed that many endogenous exosome proteins (although PM/Scl-100 was not detected in the immunoprecipitates) co-precipitated with both PM/Scl-75c-α and PM/Scl-75c-αΔNLS, but not with PM/Scl-75a-α (Figure 4a). In a similar type of experiment we demonstrated that both the α and β form of PM/Scl-75c can be incorporated in the exosome complex (Figure 4b).

Previously, we demonstrated that PM/Scl-75a-α was not able to interact directly with any other exosome component in a mammalian two-hybrid system, although its yeast counterpart (Rrp45p) is known to interact with yeast Rrp41p (106). The full-length cDNAs encoding PM/Scl-75a-α, PM/Scl-75c-α and 10 (putative) human exosome components (hRrp4p, hRrp40p, hRrp41p, hRrp42p, hRrp46p, PM/Scl-100, hCsl4p, hRrp44p/hDis3p, OIP2 and hMtr3p) were cloned in both the pACT (in-frame with the sequence encoding the VP16 transcription

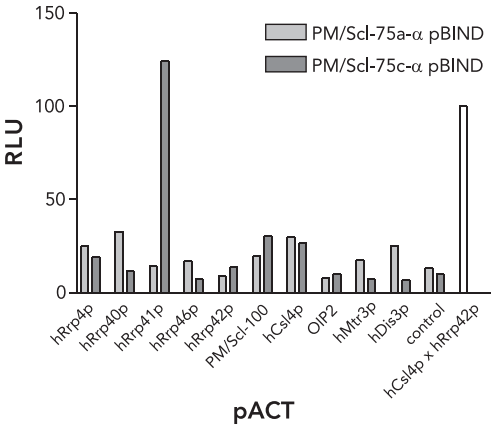


**Figure 5. Two-hybrid interactions between PM/Scl-75a-α, PM/Scl-75c-α and other exosomal proteins.**
COS-1 cells were cotransfected with constructs encoding PM/Scl-75a-α, PM/Scl-75c-α and other exosomal proteins, fused to either the VP16 transcription activation domain (pACT constructs) or the GAL4 DNA binding domain (pBIND constructs), simultaneously with a reporter plasmid. The resulting luciferase activity is depicted in relative luminescence units (RLU). The activity observed for the combination of hRrp42p in pBIND and hCsl4p in pACT was defined as 100 RLU (88).

activation domain) and pBIND (in-frame with the sequence encoding the GAL4 DNA binding domain) vectors of the Checkmate Mammalian Two-Hybrid system. COS-1 cells were co-transfected with each pair of these constructs and after 40-48 hours the luciferase activity of extracts prepared from these cells was determined. This revealed that, in contrast to PM/Scl-75a-α, PM/Scl-75c-α fused to the GAL4 DNA binding domain was able to interact with hRrp41p fused to the VP16 activation domain (Figure 5). This interaction was confirmed by a complementary experiment in which the fusion domains were exchanged (data not shown). The interaction of PM/Scl-75c with hRrp41p adds further evidence to the model for the human exosome, in which PM/Scl-75 is flanked by hRrp41p and hRrp46p (106).

### *Alternative splicing of PM/Scl-75*

As can be seen in Figures 3 and 4, the subcellular localization and exosome association of the PM/Scl-75c-β isoform, containing the extra exon 10⋆, in living cells was indistinguishable from that of the PM/Scl-75c-α isoform. The data from the EST libraries (see above) suggested that the α isoform is more abundantly expressed than the β isoform. To shed more light on this issue, we studied their expression in several cell lines by western blotting. The ratio between the amounts of the α and β isoforms appeared to vary significantly between different cell lines, although in most cells analyzed PM/Scl-75-α was at least as abundant as PM/Scl-75-β. Figure 6 shows that in Jurkat, HeLa and HEp-2 cells (lanes 1-3) the amount of both isoforms is comparable. In COS1 and 293 cells (lanes 4-5) the α isoform of PM/Scl-75 appears to be the most abundant one. In HeLa cells, the relative level of PM/Scl-75-β was significantly higher in nuclear extracts than in cytoplasmic extracts (lanes 6-7), suggesting a predominant nuclear localization for PM/Scl-75-β.

## Discussion

In this study we have shown that the previously reported cDNA and amino acid sequences for the human polymyositis/scleroderma autoantigen PM/Scl-75 are probably incomplete. We cloned a cDNA with an extended N-terminus and only the longer form of PM/Scl-75 was able to associate with the exosome complex, to interact with at least one other exosome subunit and to enter the nucleolus, three functional activities that are shared with other exosome subunits. The presence of exon 10⋆ in the coding sequence of PM/Scl-75 (observed in a previously reported splice variant) did not affect the behavior of PM/Scl-75 with regard to its subcellular localization and exosome association. In addition, PM/Scl-75 was shown to contain both N- and C-terminal

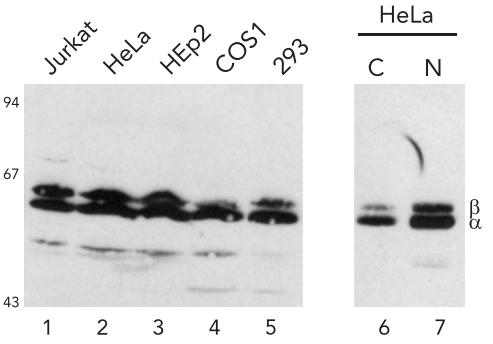


**Figure 6. The PM/Scl-75-α and β splice variants are expressed in various cell lines.**
Western blots containing extracts of several cell lines were stained with rabbit anti-PM/Scl-75 antibodies. Total cell extracts of Jurkat, HeLa, HEp2, COS1 and 293 cells are shown in lanes 1-5. Lanes 6 and 7 contain HeLa cytoplasmic and nuclear extract, respectively. The positions of molecular weight markers are indicated on the left and the positions of the α and β isoforms on the right.

elements involved in its nuclear localization, the latter of which also appeared to be responsible for nucleolar accumulation of this protein.

*The amino acid sequence of PM/Scl-75*

The cDNA of PM/Scl-75a-α (8) lacks the first exon compared to the cDNA of PM/Scl-75c-α and as a consequence the open reading frame starts at another methionine. Note that the 5' end of the PM/Scl-75b-α cDNA is identical to that of PM/Scl-75a-α, with the exception of one additional nucleotide in the former, leading to a longer open reading frame in the 5' region (122). The expression of mRNAs corresponding to these two types of cDNAs is most likely due to the existence of alternative promoters and transcription initiation sites in the gene of this protein (see Figure 1C). In contrast to PM/Scl-75c-α the expression of PM/Scl-75a-α and PM/Scl-75b-α mRNAs is not supported by entries in the EST databases. A TBLASTN search of the human EST database with the N-terminal 80 amino acids of the mouse sequence resulted in 49 out of 50 hits that fully matched the N-terminus of the additional sequence. No ESTs were observed encoding the sequence corresponding to the N-terminus of PM/Scl-75a or PM/Scl-75b. Nevertheless, the latter was detected in several cell lines by RT-PCR, followed by sequencing of the products.

The lack of expression of PM/Scl-75a-α is supported by the observation that the corresponding in vitro translated protein migrates faster than the PM/Scl-75 protein from HeLa cells (Figure 2) (8). However, immunoblotting of primate cell extracts with rabbit anti-PM/Scl-75 antibodies revealed several minor polypeptide bands, including one with an apparent molecular mass of approximately 60 kD (Figures 2 and 6), which is similar to the migration of PM/Scl-75a-α. Taken together, these data suggest that the N-terminal region of the major PM/Scl-75 isoform corresponds to the sequence encoded by PM/Scl-75c-α and that as a result of an alternative promoter PM/Scl-75b-α is produced. The expression of mRNAs either containing or lacking exon 10* (Figure 1D) is supported by a series of database entries and thus is consistent with the expression of two splicing variants (designated α and β). Western blot analyses of extracts of several primate cell lines indeed show a doublet for PM/Scl-75, in agreement with the simultaneous expression of these splice variants in a variety of cells. Interestingly, the results of HeLa cell fractionation experiments suggested that the β isoform might accumulate somewhat more efficiently in the nucleus than the α isoform.

*Transport of PM/Scl-75 to nucleus and nucleolus*

Previously, the C-terminal element of PM/Scl-75, rich in basic amino acids, was proposed to be a nuclear localization sequence. Here, we have shown that this element indeed can direct a reporter protein to the nucleus. However, this element did not appear to be essential for nuclear import of PM/Scl-75, as long as PM/Scl-75 was able to interact with the rest of the exosome complex. On the other hand, the basic sequence element of PM/Scl-75 is important for nucleolar accumulation of this protein. Based upon the presence of putative NLS sequences in at least two other exosome proteins, hRrp41p (aa 85-90, 'ERKRRP') and PM/Scl-100 (aa 752-758, 'AKKRERA') two mechanisms for nuclear entry of the exosome can be envisaged. First, the nuclear import of the completely assembled exosome may be mediated by the concerted action of

several signals. Second, partially assembled parts of the exosome may enter the nucleus and assemble into a complete exosome in the nucleoplasm or nucleolus. All these basic elements may also be involved in nucleolar targeting of the exosome, because elements rich in basic residues have been demonstrated to play an essential role in nucleolar accumulation (123).

*Association of PM/Scl-75 with the exosome*

The direct interaction between PM/Scl-75c and hRrp41p supports the model for the human exosome that we recently proposed, as illustrated in Figure 7 (106). The minor isoform of the protein, PM/Scl-75b, most likely is also capable of interacting with hRrp41p and the exosome, because it contains the complete RNase PH domain (Figure 1b). Another interaction predicted by that model, between PM/Scl-75 and hRrp46p (Figure 7), could not be detected in the mammalian two-hybrid system. PM/Scl-75 represents together with hRrp41p the equivalent of one PNPase subunit in the structurally related PNPase trimer and the two RNase PH domains of PNPase interact with each other via their most C-terminal sequences. The failure of PM/Scl-75 to interact with its putative neighbor hRrp46p, which is predicted to be mediated by the N-terminal regions of their RNase PH domains, might be due to the fact that also the hRrp46p cDNA that was used is incomplete. Very recently an alternative sequence for hRrp46p was published (80), which contains an N-terminus with 33 additional amino acids. Although the complete RNase PH domain is present in the original sequence of hRrp46p, it is possible that some flanking amino acids might be required for the interaction with PM/Scl-75, either by making direct contacts or by stabilizing the proper conformation of the RNase PH domain. Another explanation might be the possible interference of the fusion parts of the mammalian two-hybrid constructs, which are both attached to the N-terminus of the proteins to be analyzed. Whether the AU binding properties of PM/Scl-75 (19) are also influenced by the extra N-terminal sequence remains to be investigated, although this activity is most likely mediated by the C-terminal part of the protein, because the RNase PH domain is responsible for the interaction of the protein with the exosome complex. Since no functional differences were found between the splice variants PM/Scl-75-α and PM/Scl-75-β and both PM/Scl-75b and PM/Scl-75c contain the complete RNase PH domain, the function of this multiplicity of PM/Scl-75 splice variants remains to be identified, although the function of the β form might be specific for the nucleus given its increased nuclear accumulation.

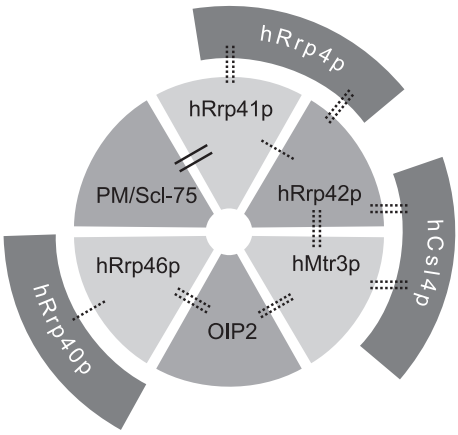


**Figure 7. Model of the human exosome and the identified protein-protein interactions.**
This model is based on previously described protein-protein interactions (dotted lines) (106) and the interactions of PM/Scl-75 identified in this study (solid lines). Double lines indicate interactions identified in both directions in the two-hybrid system.

## Materials and Methods

### *cDNA cloning and construction of deletion mutants*

All cDNAs used were cloned into pACT and pBIND vectors (Promega) or suitable pEGFP vectors (Clontech) by PCR or via available restriction sites. The database accession numbers of the cDNAs used are BC000747 (hRrp4p), AF281132 (hRrp40p), AF281133 (hRrp41p), D29958 (hRrp42p), AF281134 (hRrp46p), M58460 (PM/Scl-75a), L01457 (PM/Scl-100), AF151866 (hCsl4p), R27667 (hDis3p), AF025438 (OIP2) and NM_058219 (hMtr3p). The sequences of the cDNAs encoding PM/Scl-75c-α and PM/Scl-75c-β were submitted under accession numbers AJ505989 and AJ517294, respectively. Deletion mutants of PM/Scl-75 were constructed using suitable internal restriction sites.

### *Bioinformatics*

Predictions of functional domains and putative nuclear localization signals (NLSs) were done using the Pfam database of Hidden Markov Models (96) and the PredictNLS server (121), respectively. Sequence database searching was performed using BLAST on GenBank databases containing either ESTs or genomic sequence information.

### *Immunoblot analysis*

For immunoblot analysis, proteins were separated by SDS-PAGE, and transferred to nitrocellulose membranes. To visualize the proteins, blots were incubated with autoimmune patient or rabbit sera, diluted 5000- and 500-fold, respectively, in blocking buffer (4% skimmed milk, phosphate-buffered saline (PBS), 0.1% NP-40). As secondary antibody, horseradish peroxidase-conjugated rabbit-anti human IgG or swine anti-rabbit IgG (Dako Immunoglobulins) were used, 5000-fold diluted in blocking buffer. Visualization was performed by chemiluminescence.

### In vitro *translation*

Radioactively labeled proteins were produced using the Reticulocyte Lysate System (Promega) according to the manufacturer's procedure, in a total volume of 25 µl, containing approximately 1 µg of circular plasmid DNA (pCI-neo, Promega), containing the coding sequence of the protein, and in the presence of [$^{35}$S]methionine.

### *Reverse transcriptase-polymerase chain reaction (RT-PCR)*

RNA was isolated from cells using Trizol reagent (Gibco BRL) according to the manufacturers instructions. Synthesis of cDNA from 1 µg of RNA was performed using the Reverse Transcription System (Promega) with 10 pmol of PM/Scl-75 specific primer. The reaction mixture was incubated for 10 min at room temperature, followed by 90 min at 42°C. For RT-PCR 1 µl cDNA was added to 49µl mastermix containing 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween-20, 2 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol forward and reverse primer and 8 U Taq DNA polymerase. PCR was performed using a T3 thermocycler (Biometra): 2 min 94°C, 35 cycles of (30 s, 94°C; 30 s, 60°C; 30 s, 72°C), 5 min 72°C. 10 µl PCR product was analyzed on a 1.5% agarose gel. The following primers were used: RT primer 5'-CAGGTGTAGAAACAACTTCTGAAGGAGG-3'; PM/Scl-75 reverse primer 5'-CACCAAGAGATCTGACTGCCTGCCAG -3'; PM/Scl-75a forward primer 5'-AGATCTCGAGCCTGTATGGGCGGGCTGGTTAGGATTC-3'; PM/Scl-75c forward primer 5'-AGATCTCGTCGACCGAATTCCATGAAGGAAACGCCACTC-3'.

### *Transient transfection of HEp-2 cells and direct immunofluorescence*

For transfection, cDNAs were cloned into suitable pEGFP vectors (Clontech), allowing expression of the proteins fused to the C-terminus of the EGFP protein. HEp-2 (human epithelioma, ATCC No.: CCL-23) cells were grown to 80% confluent monolayers by standard tissue

culture techniques in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc.) containing 10% fetal calf serum (FCS). For immunoprecipitation, approximately 10 x $10^6$ cells were transfected with 20-30 µg of DNA in 1600 µl of DMEM containing 10% FCS by electroporation, which was performed at 270V and 950 µF using a Gene-Pulser II (Bio-Rad). After transfection, cells were seeded in 75-$cm^2$ culture flasks and cultured overnight. After washing twice with PBS, the cells were resuspended in 500 µl of lysis buffer (25 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM EDTA, 1 mM DTE, 0.5 mM PMSF and 0.05% NP-40) and homogenized by sonication. For fluorescence microscopy, approximately 2 x $10^6$ cells were transfected with 10-20 µg of DNA in 800 µl of DMEM containing 10% FCS by electroporation, as described above. After transfection, cells were seeded onto coverslips and cultured overnight. Cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, washed in PBS twice, briefly rinsed in acetone, dried and finally mounted with PBS/glycerol. The expressed EGFP-tagged proteins were visualized by fluorescence microscopy.

*Immunoprecipitation*

Polyclonal antibodies from rabbits and patients were coupled to protein A-agarose beads (Biozym) in IPP500 (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40) by incubation for 2 h at room temperature. Beads were washed twice with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40). For each immunoprecipitation, cell extract was incubated with the antibody-coupled beads for 1 h at 4°C. Subsequently, beads were washed three times with IPP150, and the co-immunoprecipitated proteins were analyzed by immunoblotting.

*Mammalian two-hybrid analysis*

All interactions were analyzed using the CheckMate Mammalian Two-Hybrid System (Promega) essentially according to the manufacturer's protocol. Briefly, 3-4 x $10^5$ COS-1 cells seeded in one well of a 6-wells plate were transfected with 3 vectors (1 µg each), pACT and pBIND, either with or without insert, and the pG5*luc* reporter vector using 5 µl Fugene Transfection Reagent (Roche), as described by the manufacturer. After 40-48 hours of growth, cells were harvested using 500 µl Passive Lysis Buffer (PLB; Promega) and the activity of both the firefly luciferase and the control Renilla luciferase were determined using the Dual Luciferase Reporter Assay System (Promega) on a Berthold Lumat LB 9507 Luminometer. In brief, 100 µl of firefly luciferase substrate solution was added to 20 µl of cell extract and the luminescence was measured to determine the efficiency of the interaction. Next, 100 µl of Renilla luciferase substrate solution (containing a quencher for the firefly luciferase activity) was added and again the luminescence was determined to monitor the transfection efficiency.

# Chapter 6

## Caspase-mediated cleavage of the exosome subunit PM/Scl-75 during apoptosis

Reinout Raijmakers[1]
Kelen C.R. Malmegrim de Farias[1,2]
Xavier Saelens[3]
Wilma Vree Egberts[1]
Walther J. van Venrooij[1]
Peter Vandenabeele[3]
Ger J.M. Pruijn[1]

[1] *Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, Nijmegen, The Netherlands*

[2] *Department of Clinical Medicine, Division of Clinical Immunology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil*

[3] *Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology and Ghent University, Ghent, Belgium*

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*Recent studies have implicated the dying cell as a potential reservoir of modified autoantigens that might initiate and drive systemic autoimmunity in susceptible hosts. A number of subunits of the exosome, a complex of 3′ → 5′ exoribonucleases which functions in a variety of cellular processes, are recognized by the so-called anti-PM/Scl autoantibodies, found predominantly in patients suffering from an overlap syndrome of myositis and scleroderma. Here we show that one of these subunits, PM/Scl-75, is proteolytically cleaved in apoptotic cells. Cleavage of the PM/Scl-75 protein occurs in the C-terminal part of the protein, leaving the more N-terminally located RNase PH domain intact. The N-terminal fragment of PM/Scl-75 remains associated with the exosome complex in apoptotic cells. PM/Scl-75 cleavage is dependent on caspase activation, but the exact cleavage site(s) remain to be identified. The analysis of PM/Scl-75 cleavage by recombinant caspase proteins and the inhibition of cleavage by different caspase inhibitors indicates that PM/Scl-75 is a substrate of different caspases, including caspase-1 and caspase-8, both in vitro and in vivo. The implications of PM/Scl-75 cleavage for exosome function and the generation of anti-PM/Scl-75 autoantibodies are discussed.*

## Introduction

Systemic autoimmune diseases are characterized by the presence of autoantibodies reactive with a wide variety of autoantigens. Why these autoantibodies, which escape the normal mechanisms ensuring self tolerance, are made is still not fully understood. However, the occurrence of modified self-antigens during (either apoptotic or necrotic) cell death in combination with a defective clearance of dead cells has been proposed to play a role in the development of autoimmunity (reviewed in (124,125)). Many autoantigenic proteins or complexes have been shown to be modified in dying cells. The modification of these proteins, for example specific cleavage by caspases, might generate antigenic determinants to which no tolerance exists, thereby eliciting a primary immune response. Via epitope spreading, the initial response, directed to the neo-epitope resulting from the modification, could evolve to a secondary response in which antibodies arise that are reactive with other, unmodified parts of the protein or with proteins that are associated with the modified antigen (124,125). Besides proteolytic cleavage by caspases, a number of other apoptotic and necrotic modifications have been observed in known autoantigens, including (de)-phosphorylation, citrullination and RNA cleavage (reviewed in (126)).

Patients suffering from myositis and scleroderma (Scl), which is called the polymyositis/scleroderma overlap syndrome (PM/Scl), produce antibodies against a variety of autoantigens. Some of these are also found in patients suffering from myositis or scleroderma alone. Autoantibodies recognizing the so-called PM/Scl autoantigen are found in approximately 24% of all patients with PM/Scl (2,68,69), and in only 2-6% of patients suffering from myositis or scleroderma alone (68,77). Of all patients positive for anti-PM/Scl antibodies, between 43% and 88% are diagnosed

with a myositis/scleroderma overlap syndrome (68,73). The PM/Scl autoantigen consists of a multiprotein complex representing the human homologue of the yeast exosome, which contains at least ten proteins, all displaying exoribonuclease characteristics. The exosome has been shown to be involved in the degradation and processing of many different RNA species (11). Although many of the proteins associated with the human exosome complex are to some extent autoantigenic (79), three of the proteins carry the main autoantigenic epitopes, PM/Scl-100, PM/Scl-75 and hRrp4p (7-9,79). Until now, none of the subunits of the exosome complex had been shown to be modified during apoptosis, prompting us to investigate the behaviour of exosome subunits after induction of apoptosis. Here, we show that the PM/Scl-75 protein is cleaved in a caspase dependent manner during apoptosis and that this cleavage occurs in the C-terminal half of the protein.

## Results

### *Cleavage of PM/Scl-75 during apoptosis*

To study the potential modification of human exosome components during apoptosis, Jurkat cells were treated with the agonistic anti-Fas antibody 7C11 and cell extracts were analyzed by western blotting using a patient serum (Ven96) known to be reactive with PM/Scl-100, PM/Scl-75 and hRrp4p. As can be seen in Figure 1A, the intensity of the PM/Scl-75 band was strongly reduced after the induction of apoptosis, whereas

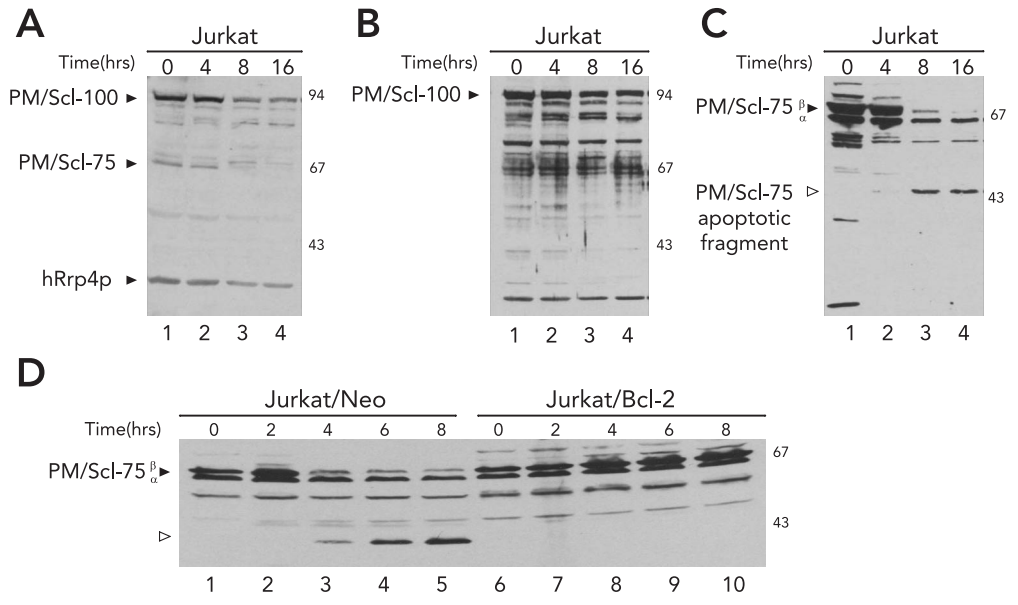


**Figure 1. Cleavage of the PM/Scl-75 protein during anti-Fas induced apoptosis.**
Jurkat cells were treated with the anti-Fas monoclonal antibody 7C11 for the indicated time periods. Protein extracts were analyzed by 10% SDS-PAGE and western blotting using the anti-PM/Scl patient serum Ven96 (A), the anti-PM/Scl-100 polyclonal rabbit serum (B) or the anti-PM/Scl-75 polyclonal rabbit serum (C). Jurkat/Neo and Jurkat/Bcl-2 were treated with the anti-Fas monoclonal antibody 7C11 for the indicated time periods. Protein extracts were analyzed by 10% SDS-PAGE and western blotting using the anti-PM/Scl-75 polyclonal rabbit serum (D). In each panel, the positions of molecular weight markers are indicated on the right and the positions of the relevant polypeptides on the left. Closed arrowheads indicate full-length proteins and open arrowheads indicate cleavage products.

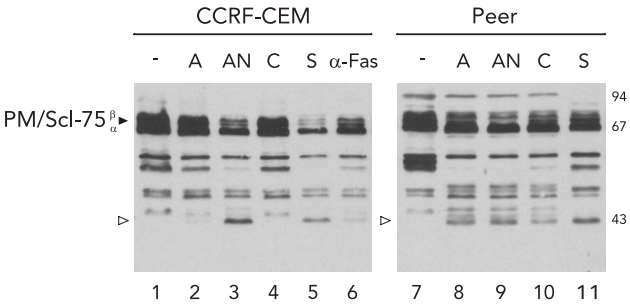


**Figure 2. Cleavage of the PM/Scl-75 protein in cells treated with different apoptosis inducers.**
Cells (CCRF-CEM and Peer) were treated with anti-Fas mAb 7C11 (α-Fas), actinomycin D (A), anisomycin (AN), cycloheximide (C), or staurosporin (S). Cell extracts were analyzed by 10% SDS-PAGE and Western blotting using the anti-PM/Scl-75 polyclonal rabbit serum. The positions of molecular weight markers are indicated on the right and the positions of the relevant polypeptides on the left. Closed arrowheads indicate full-length proteins and open arrowheads indicate cleavage products.

the level of PM/Scl-100 and hRrp4p were only slightly diminished. To analyze this phenomenon in more detail, identical blots were analyzed with rabbit sera recognizing either PM/Scl-100 or PM/Scl-75 (Figures 1B and 1C, respectively). These results confirmed that the majority of full-length PM/Scl-100 polypeptides remains intact during apoptosis (Figure 1B). However, PM/Scl-75, in particular the β splice variant of this protein, disappeared during apoptosis, concomitant with the appearance of a specific smaller fragment migrating at approximately 45 kDa (Figure 1C). To investigate whether this apparent PM/Scl-75 cleavage was indeed dependent on the induction of apoptosis, the cleavage of PM/Scl-75 was analyzed in anti-Fas stimulated Jurkat cells (Jurkat/Neo) and cells overexpressing Bcl-2 (Jurkat/Bcl-2). In the Jurkat/Bcl-2 cells the induction of apoptosis has been demonstrated to be delayed considerably (127). Indeed, the PM/Scl-75 cleavage product was present in the Jurkat/Neo cells, but could not be detected in de Jurkat/Bcl-2 cells, not even after 8 hours of anti-Fas treatment (Figure 1D).

To investigate whether cleavage of PM/Scl-75 also occurred in cells exposed to other apoptotic stimuli, we analyzed lysates of other cell lines in which apoptosis had been induced in various ways. CCRF-CEM cells were exposed to actinomycin D, anisomycin, cycloheximide, staurosporin or anti-Fas antibodies and Peer cells were cultured in the presence of actinomycin D, anisomycin, cycloheximide or staurosporin, and after 8 hours extracts of these cells were analyzed by western blotting (Figure 2). Except for the actinomycin D (lane 2) and cycloheximide (lane 4) treated CCRF-CEM cells, the cleavage product of PM/Scl-75 was detected in all lysates, indicating that PM/Scl-75 cleavage is a general phenomenon in apoptotic cells. The anti-PM/Scl-75 rabbit serum used to stain the protein also showed reactivity with a number of yet unidentified proteins, including a protein of about 50 kDa, that also disappears upon the induction of apoptosis.

### *The PM/Scl-75 cleavage product remains associated with the exosome complex*

To analyze if the apoptotic cleavage of PM/Scl-75 could occur when the protein is associated with the exosome complex, immunoprecipitations were performed using extracts from control and apoptotic Jurkat cells. Figure 3 shows that not only anti-PM/Scl-75 rabbit sera and patient serum Ven96, but also rabbit antibodies to hRrp46p, another exosome subunit, can precipi-

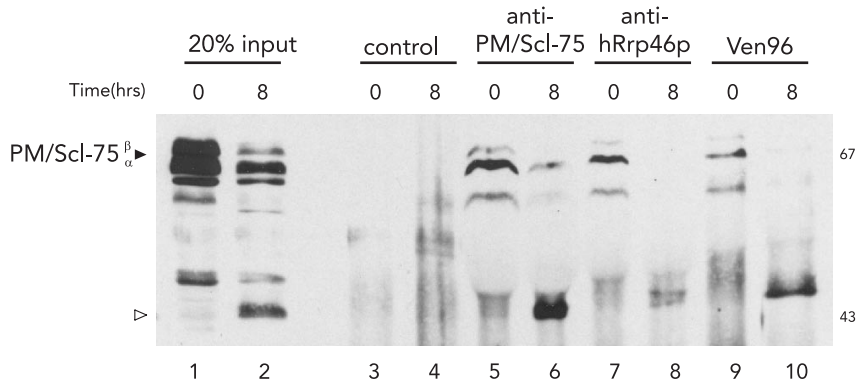


**Figure 3. The apoptotic cleavage product of PM/Scl-75 remains associated with the exosome.**
Immunoprecipitations from control and apoptotic (8 h incubated with anti-Fas antibody) Jurkat cell extracts were performed using normal rabbit serum (lanes 3 and 4), anti-PM/Scl-75 (lanes 5 and 6) and anti-hRrp46p (lanes 7 and 8) polyclonal rabbit serum, and patient serum Ven96 (lanes 9 and 10). Immunoprecipitates were analyzed by 10% SDS-PAGE and Western blotting using anti-PM/Scl-75 polyclonal mouse serum. The positions of the PM/Scl-75 protein (closed arrowhead) and its cleavage product (open arrowhead) are indicated on the left. In lanes 1 and 2 input samples, 20% of the total control and apoptotic cell extracts, respectively, were loaded

tate both the wild-type and the cleaved PM/Scl-75 protein. This indicates that PM/Scl-75 can be cleaved while it is associated with hRrp46p and probably the entire exosome and suggests that cleavage does not abrogate its association with the complex.

*PM/Scl-75 cleavage is caspase mediated*

Because the activation of caspases is a common feature of apoptotic cells and because activated caspases are capable of cleaving a large number of (autoantigenic) proteins, it was investigated whether caspase activation was required for cleavage of PM/Scl-75. Jurkat cells were cultured

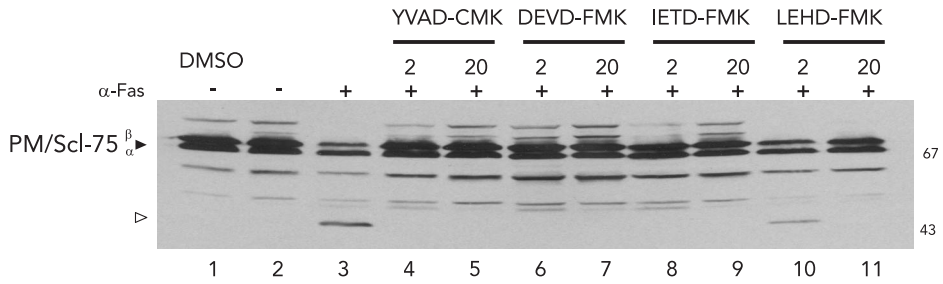


**Figure 4. Inhibition of PM/Scl-75 cleavage by caspase inhibitors.**
Jurkat cells were incubated for 1h at 37 °C in the presence of four cell permeable tetrapeptide caspase inhibitors: 2 μM (lanes 4, 6, 8, 10) or 20 μM (lanes 5, 7, 9, 11) of inhibitors for caspase-1 (YVAD-CMK), caspase-3 (DEVD-FMK), caspase-8 (IETD-FMK) and caspase-9 (LEHD-FMK). Subsequently, cells were cultured for 8 hours in the presence of anti-Fas mAb. In lane 1 control extract from mock-treated Jurkat cells was loaded. In lanes 2 and 3 extracts from control and apoptotic (8 h, anti-Fas mAb) Jurkat cells, respectively, were analyzed. Total protein extracts were analyzed by 10% SDS-PAGE and western blotting using the anti-PM/Scl-75 polyclonal rabbit serum. The positions of molecular weight markers are indicated on the right and the positions of the relevant polypeptides on the left. Closed arrowheads indicate full-length proteins and open arrowheads indicate cleavage products.

in the presence of various inhibitors of caspases for 1 hour prior to the induction of apoptosis by anti-Fas. Four different tetrapeptide caspase inhibitors were used, Ac-YVAD-CMK for group I caspases (caspase-1-like), Z-DEVD-FMK for group II caspases (caspase-3-like), or Z-IETD-FMK (caspase-8) and Z-LEHD-FMK (caspase-9) for group III caspases. Rabbit anti-PM/Scl-75 antiserum was used to analyze the cleavage of PM/Scl-75 in these cells. The cleavage of PM/Scl-75 was completely inhibited in the presence of 2 or 20 μM of Ac-YVAD-CMK, Z-DEVD-FMK, or Z-IETD-FMK inhibitors (Figure 4, lanes 4–9). In the presence of the Z-LEHD-FMK inhibitor, PM/Scl-75 cleavage was partially inhibited at 2 μM and completely inhibited in the presence of a 10-fold higher concentration (Figure 4, lanes 10–11). These results suggest that caspases, or other proteases activated downstream of the caspase-cascade, are involved in the apoptotic cleavage of PM/Scl-75.

### *Cleavage of PM/Scl-75 by different caspases*

To determine whether PM/Scl-75 is a direct substrate for recombinant caspases and, if so, whether the major cleavage product had a similar electrophoretic migration behaviour as the cleavage product observed in cell extracts, *in vitro* translated PM/Scl-75a-α and PM/Scl-75a-β (see also Figure 6) were incubated with recombinant murine caspases-1, -2, -3, -7, -8 and -11. Caspase-1 very efficiently cleaved PM/Scl-75 (Figure 5A, lanes 2, 9), whereas cleavage, although to a lesser extent, was also observed with caspase-8

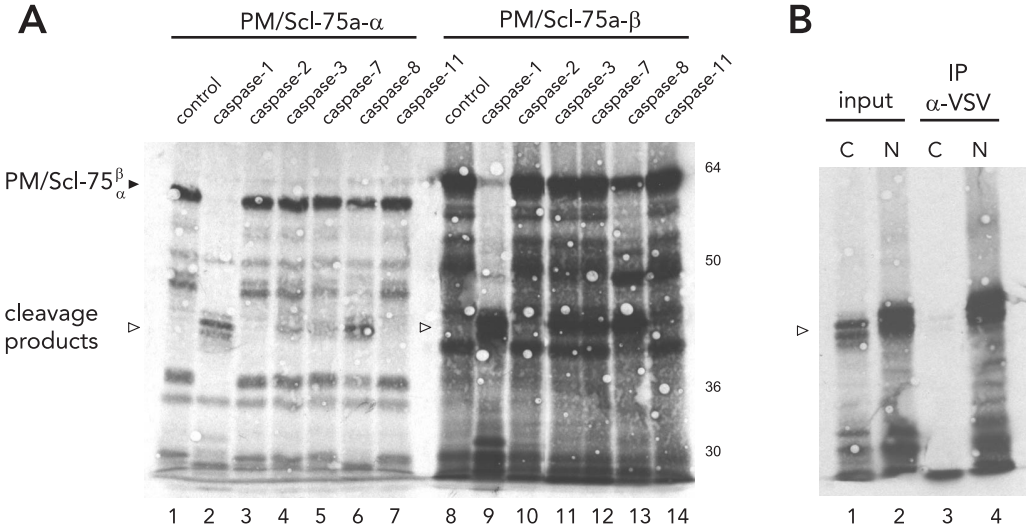


**Figure 5. Cleavage of PM/Scl-75 by recombinant caspases.**
In vitro translated $^{35}$S-labeled PM/Scl-75a-α and PM/Scl-75a-β were incubated with 200 nM of purified murine recombinant caspases-1, -2 , -3, -7, -8 or -11 for 1.5 h at 37°C. The resulting reaction products were analyzed by 10% SDS-PAGE, followed by autoradiography (A). In the first lane the mock-incubated protein was loaded. Closed arrowheads mark the full-length proteins and open arrowheads mark the cleavage products. Immunoprecipitations were performed with anti-VSV-tag antibodies using either C- or N-terminally VSV-tagged PM/Scl-75a-α (lanes 3 and 4), after cleavage of the protein with recombinant caspase-1 as in panel A. The input of the respective immunoprecipitation are loaded in lanes 1 and 2 (B). In each panel, the positions of molecular weight markers are indicated on the right and the positions of the relevant polypeptides on the left. Closed arrowheads indicate full-length proteins and open arrowheads indicate cleavage products.

(lanes 6, 13) and caspases-3 and -7 (lanes 4, 5, 11, 12). Interestingly, a number of cleavage products appeared, displaying small differences in electrophoretic mobility, which suggests the presence of a number of different cleavage sites. The cleavage products observed for PM/Scl-75a-α (Figure 5A, lanes 1-7) and PM/Scl-75a-β (lanes 8-14) appeared to be of identical size, suggesting that these PM/Scl-75 fragments lack the C-terminal region of the protein, because this region contains the sequence encoded by the extra exon present in the β variant. Since caspase-8 plays a key role in death domain receptor mediated apoptosis, it was tested whether caspase-8 is essential for the cleavage of PM/Scl-75 in apoptotic cells. Therefore, caspase-8-deficient Jurkat cells (ATCC CRL-2571) were used to analyze the apoptotic processing of PM/Scl-75. These cells become apoptotic when treated with staurosporin in combination with cycloheximide. Despite the absence of caspase-8, upon induction of apoptosis the same cleavage pattern was observed as in control cells containing caspase-8 (data not shown). This suggests that, in agreement with the *in vitro* proteolysis results, other caspases might be responsible for cleavage of PM/Scl-75 in apoptotic cells.

### *Putative cleavage sites in PM/Scl-75*

Using recombinant capase-1, *in vitro* translated PM/Scl-75a-α carrying a VSV-G-tag at either the N- or the C-terminus of the protein was cleaved. Next, immunoprecipitations were performed, using an anti-VSV-tag monoclonal antibody. As is shown in Figure 5B, the cleavage products of PM/Scl-75 could only be immunoprecipitated when the VSV-tag was attached to the N-terminus of the protein, confirming that the major cleavage products observed by SDS-PAGE contain the N-terminal, but not the C-terminal part of PM/Scl-75.

These data for the *in vitro* cleavage products are in agreement with the *in vivo* cleavage data,

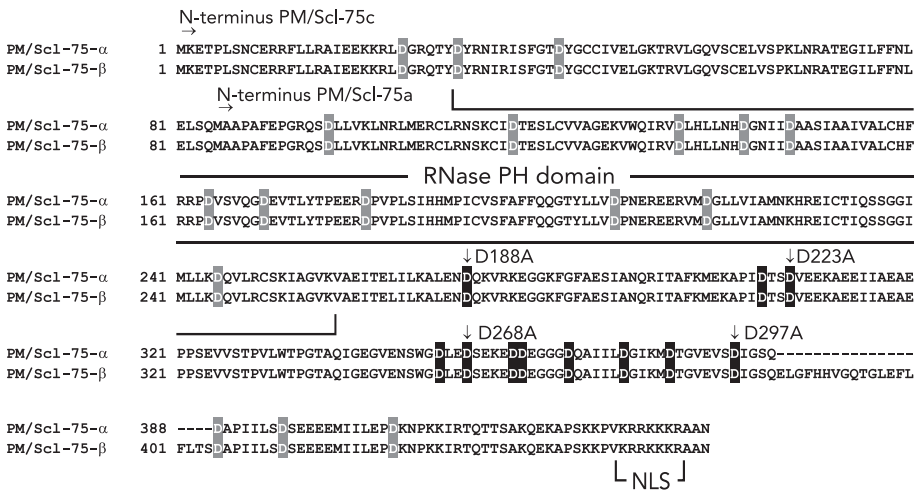


**Figure 6. Amino acid sequence of PM/Scl-75.**
An alignment of both splice variants (α and β) of PM/Scl-75 is shown, in which the RNase PH domain, the NLS and the N-terminus of both published sequences for the protein, PM/Scl-75a (8) and PM/Scl-75c (129), are indicated. In addition, all aspartic acid residues are highlighted in black if they are putative cleavage sites based on available data, and otherwise in gray. Finally, the aspartic acid residues that were mutated in an attempt to find the cleavage site are marked.

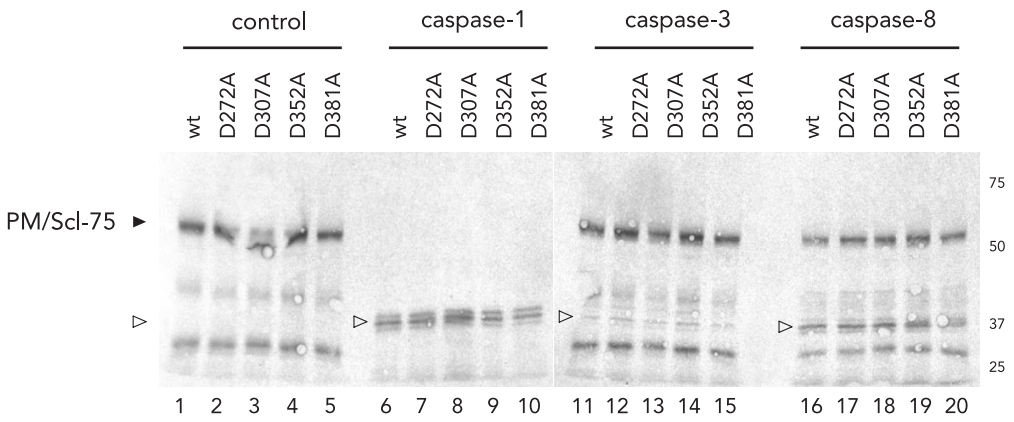


**Figure 7. Cleavage of PM/Scl-75 mutants by recombinant caspases.**
*In vitro* translated $^{35}$S-labeled PM/Scl-75a-α and mutants of the protein in which the aspartic acid residues at positions 272 (D272A), 307 (D307A), 352 (D352A) or 381 (D381A) were changed into alanine residues, were incubated with 200 nM of purified murine recombinant caspases-1, -3, or -8 for 1.5 h at 37°C. The resulting reaction products were analyzed by 10% SDS-PAGE, followed by autoradiography. In the lanes 1-5 the mock-incubated proteins were loaded. The positions of molecular weight markers are indicated on the right and the positions of relevant polypeptides on the left. Closed arrowheads indicate full-length proteins and open arrowheads indicate cleavage products.

because the major product observed *in vivo* was shown to be associated with the exosome complex (Figure 3) and therefore still contains the N-terminal part of the protein which encompasses the RNase PH domain, which is required for the interaction with other exosome subunits. Taken together these data indicate that the caspase cleavage site(s) in PM/Scl-75 are located in the C-terminal half of the protein, C-terminal from the RNase PH domain and N-terminal from the sequence encoded by the PM/Scl-75a-β specific exon (Figure 6).

Based on homology with previously described caspase-1 and -8 cleavage sites, four aspartic acid residues were selected that might represent caspase cleavage sites in PM/Scl-75. To investigate their involvement in cleavage, the aspartic acid residues at position 272 ('LEN*D*'), 307 ('DTS*D*'), 352 ('DLE*D*') and 381 ('EVS*D*') were substituted by alanine and the corresponding *in vitro* translated mutants of PM/Scl-75 were incubated with caspases-1, -3 and -8. In Figure 7, it can be seen that all four mutants were still cleaved by these caspases, suggesting that at least *in vitro* these aspartic acid residues are not essential for caspase mediated cleavage of PM/Scl-75.

## Discussion

Although many different autoantibodies have been identified in a variety of autoimmune diseases, the origin of these autoreactive antibodies is still unknown. During the last decade a substantial number of autoantigens have been shown to be modified during apoptosis and/or necrosis. This has led to the hypothesis that intracellular, modified autoantigens are exposed to the immune system because of massive cell death and/or inefficient removal of dying cells, which could elicit a primary immune response targeting the modification on the autoantigen. Subsequently, in a secondary response, involving epitope spreading, also other parts of the autoantigen will be targeted by the immune system (124). Patients

with the PM/Scl overlap syndrome often develop antibodies against a number of components of the human PM/Scl or exosome complex, especially PM/Scl-100, PM/Scl-75 and hRrp4p (79). Here, we show for the first time that one of the exosome subunits, PM/Scl-75, is specifically modified during apoptosis. PM/Scl-75 is cleaved, probably by one or more caspases, in the C-terminal part of the protein. The electrophoretic mobility of the products observed after cleavage with recombinant caspases (at least three individual cleavage products of similar size) suggests that the caspase cleavage sites in PM/Scl-75 are most likely located in the highly charged region between aspartic acid 349 and aspartic acid 381 (Figure 6). Strikingly, the cleavage products observed in apoptotic extracts did not show this heterogeneity, suggesting that one of the aspartic acid residues in this region serves as the predominant cleavage site in vivo. This difference between the *in vitro* and *in vivo* data might be explained by the association of PM/Scl-75 with the exosome *in vivo*. Four of the aspartic acid residues in this region of the protein (D272, D307, D352 and D381) did not serve as the predominant caspase cleavage sites *in vitro* (Figure 7). The exact position(s) where the protein is cleaved, both during apoptosis *in vivo* and *in vitro* using recombinant caspases remain to be identified. Although for most caspase proteins preferential cleavage sites have been identified, for example LE(T/V)D for caspase-8 and (W/L)EHD for caspase-1 (130), a number of autoantigens, including DNA topoisomerase 1 and Sm-F, have been shown to be cleaved at unconventional sites, making it difficult to predict the exact cleavage site in PM/Scl-75 (128,131).

Consistent with the observation that the C-terminal part of the protein is removed during apoptosis, leaving the RNase PH domain intact, PM/Scl-75 remains associated with the core of the exosome complex. Based on interactions between exosome components and structural similarity with the bacterial protein polynucleotide phosphorylase (PNPase), we have recently generated a model for the structure of the human exosome. In this model the six proteins containing an RNase PH domain (RPD) form the core of the exosome, which adopts a hexameric ring structure (106). Interestingly, this structural model suggests that the RPD of PM/Scl-75 is in tight association with other proteins, whereas the C-terminal part of the protein extends from the outer surface of the ring, leaving the putative cleavage site accessible for caspases. A very similar situation has been described for the Sm-F protein, which is apoptotically cleaved while associated with the heptameric ring of the Sm complex (128). Currently, it is not known whether the total cellular pool of PM/Scl-75 molecules is associated with the exosome complex. The possibility exists that a certain fraction of it is 'free'. The efficiency by which PM/Scl-75 is cleaved, however, suggests that if both exosome associated and 'free' pools of the protein exist, both will be cleaved in apoptotic cells.

The results of several experiments indicate that the β splice variant is more efficiently cleaved in vivo than the α isoform. This difference is most likely due to a better accessibility of the caspase cleavage site(s), because the extra sequence of PM/Scl-75-β is located near the aspartic acid-rich region. The β isoform has been implicated to be preferentially associated with the nuclear exosome complex as compared to the α isoform (129).

To assess whether the cleavage of PM/Scl-75 by caspases leads to the generation of neo-epitopes on the proteins, the exact cleavage site(s)

have to be identified, for example by mutagenesis of the aspartic acid residues in the highly charged region depicted in Figure 6. Another approach would be to investigate whether the B-cell repertoire of PM/Scl patients contains antibodies that are specifically reactive with the cleaved PM/Scl-75 protein. Autoantibodies only reactive with apoptotically modified isoforms of the autoantigen have been suggested to exist for the U1-70K protein (132). In addition to the generation of autoantigenic epitopes, cleavage of PM/Scl-75 might also have implications for the function of the protein and the function of the exosome complex. It has been shown that PM/Scl-75 contains a nuclear localization signal (NLS) close to the C-terminus of the protein, which plays a role in the nuclear and nucleolar targeting of PM/Scl-75 and possibly the entire exosome complex (129). Because cleavage of PM/Scl-75 in apoptotic cells separates the NLS containing part from the protein, the subcellular distribution of the protein and the associated complex in these cells might change. It is tempting to speculate that this leads to an increased exosome concentration in the cytoplasm and/or nucleoplasm, which may be required for an enhanced degradation rate of a variety of RNA molecules in the cytoplasm.

## Materials and Methods

### *Cell lines*

Jurkat cells (human T-cell leukemia, ATCC CRL-2570), Peer cells (human T-cell leukemia) and CCRF-CEM cells (human T-cell lymphoblastic leukemia, ATCC CCL-119) were grown in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 mM sodium-pyruvate, penicillin, and streptomycin. Jurkat cells stably transfected with Bcl-2 (Jurkat/Bcl-2) or with the empty transfection vector (Jurkat/Neo) were cultured in the same medium with the addition of 200 μg/ml G418 (Gibco-BRL).

### *Induction of cell death*

To induce apoptosis, Jurkat cells were treated with the agonistic anti-Fas monoclonal antibody 7C11 as described previously (127,128). Peer and CCRF-CEM were treated with 0.5 μg/ml actinomycin D, 10 μg/ml anisomycin, 100 μg/ml cycloheximide or 400 nM staurosporin. CCRF-CEM cells were also treated with the anti-Fas antibody. The extent of induced apoptosis was assessed by flow cytometry using annexin V-FITC and propidium iodide (PI) staining as described previously (127). After 8 h generally more than 90% of the cells were apoptotic. After harvesting the dying cells, cells were washed two times with PBS and used immediately or stored at -70° C. For experiments utilizing the cell-permeable tetrapeptide caspase inhibitors (Calbiochem), Jurkat cells were cultured for 1 h in the presence of 2 or 20 μM Ac-YVAD-CMK, Z-DEVD-FMK, Z-IETD-FMK, or Z-LEHD-FMK (irreversible inhibitors of caspases-1, -3/-7, -8, and -9, respectively) as described previously (128). The specificity of these inhibitors is based on in vitro assays with purified caspases. Their specificity in a cellular context is difficult to define. Subsequently, apoptosis was induced by the addition of anti-Fas monoclonal antibody followed by harvesting the cells after 8 h of incubation.

### *Western blot analysis*

Cells were lysed on ice for 30 min in NP-40 lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)), containing a protease inhibitor cocktail (Complete, Roche). Cell lysates were centrifuged for 15 min at 4° C (12,000g) and the supernatants were used immediately or stored at -70° C. Protein extracts of $1x10^6$ cells were analyzed by 10% SDS-PAGE and western blotting using SLE patient serum Ven96 (a patient serum reactive with many exosome subunits (88)), an anti-PM/Scl-100 rabbit serum (14) or an

anti-PM/Scl-75 rabbit serum (57), followed by detection via HRP-conjugated secondary antibodies and visualization by chemiluminescence.

### *Immunoprecipitation*

Protein A–agarose beads (20 µl of 50% slurry) were coated with 20 µl of Ven96 patient serum, anti-hRrp46p rabbit serum(14) or anti-PM/Scl-75 (57) rabbit serum. Incubations were performed overnight at 4° C in IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% NP-40) by end-over-end rotation. After washing the beads three times with IPP150, the beads were incubated with 20 µl ($2x10^6$ cell equivalents) of Jurkat extract (control or apoptotic) in IPP150 by end-over-end rotation for 2 h at 4° C. After three wash steps with IPP150, the beads were resuspended in protein sample buffer and immunoprecipitated proteins analysed by SDS-PAGE and western blotting.

### *Plasmids*

The cDNAs of PM/Scl-75a-α and PM/Scl-75a-β (129) were cloned into the EcoRI and XbaI sites of the pCI-neo vector, containing an in-frame VSV-G tag at either the 5' or the 3' end of the cDNAs. Four mutant cDNAs of PM/Scl-75a-α were generated, encoding substitution mutants in which one of the aspartic acids at positions 272, 307, 352 and 381 was substituted by alanine. All mutants were generated by a megaprimed PCR-based approach using the orginal PM/Scl-75a-α cDNA as a template and specific primers overlapping the regions that were mutated. The resulting PCR products were purified, digested with EcoRI and XbaI and inserted into the EcoRI and XbaI sites of pCI-neo. The integrity of the mutant constructs was confirmed by DNA sequencing. The resulting cDNAs were used as template for transcription/translation of the respective proteins.

### In vitro *cleavage assay*

Proteins were generated by *in vitro* transcription/translation using the TnT T7-coupled rabbit reticulocyte lysate system (Promega) as described by the manufacturer. For detection of the translation products, $^{35}$S-methionine was added to the translation reactions. In vitro translated proteins were incubated with 200 nM of the purified murine recombinant caspases in a total volume of 25 µl CFS buffer (220 nM mannitol, 68 nM sucrose, 2 mM NaCl, 2.5 mM $KH_2PO_4$, 10 nM HEPES, pH 7.4, 1 mM aprotinin, 1 mM leupeptin, and 1 mM PMSF, supplemented with 5 mM DTT) for 1.5 h at 37° C. The resulting cleavage products were analyzed by 10% SDS-PAGE, followed by autoradiography.

### *Immunoprecipitation of* in vitro *cleaved proteins*

*In vitro* translated PM/Scl-75a-α containing either an N- or an C-terminal VSV-G tag was incubated with recombinant caspase-1 and subjected to immunoprecipitation using protein G–agarose beads coated with anti-VSV-G mouse monoclonal antibody. Precipitated proteins were fractionated on 10% SDS-PAGE and visualized by autoradiography.

# Chapter 7

## PM/Scl-75 is the main autoantigen in patients with the polymyositis/ scleroderma overlap syndrome

Reinout Raijmakers[1]
Manfred Renz[2]
Claudia Wiemann[2]
Wilma Vree Egberts[1]
Hans Peter Seelig[2]
Walther J. van Venrooij[1]
Ger J.M. Pruijn[1]

[1] *Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, University of Nijmegen, Nijmegen, The Netherlands*

[2] *Institute of Immunology and Molecular Genetics, Karlsruhe, Germany*

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*Objective*
To compare the autoantigenicity of the recently described N-terminally elongated PM/Scl-75 protein with that of PM/Scl-100 and the originally defined PM/Scl-75 polypeptide and to determine its value for analyzing sera from patients with the polymyositis/scleroderma overlap syndrome.
*Methods*
Sera of patients suffering from PM/Scl overlap syndrome and several other diseases were analyzed for the presence of autoantibodies reactive with recombinant PM/Scl-100 and PM/Scl-75 (both the original and the longer form) proteins in an ELISA assay.
*Results*
Autoantibodies recognizing the longer PM/Scl-75 protein isoform were detected in 28% of the PM/Scl patients, which is slightly higher than PM/Scl-100 (25%) and significantly higher than the previously defined PM/Scl-75 protein (11%). In addition, a significant number of patients was identified that had anti-PM/Scl-75, but not anti-PM/Scl-100 antibodies, in contrast to what has been reported in the past for the shorter version of the PM/Scl-75 protein.
*Conclusion*
Our data indicate that the use of the long PM/Scl-75 isoform in addition to PM/Scl-100 in ELISA significantly increases the number of patients in which anti-PM/Scl autoantibodies can be detected.

## Introduction

Specific autoantibodies in the sera of patients with clinical symptoms of polymyositis (PM) or dermatomyositis (DM) and scleroderma (Scl), called the polymyositis/scleroderma overlap syndrome (PM/Scl), capable of precipitating antigens from calf thymus extract, were first described in 1977. The antigen recognized by these autoantibodies was shown to be a nucleolar complex, consisting of 11-16 proteins ranging from 20-110 kDa (6). These characteristics allow the detection of these antibodies in serum by indirect immunofluorescence (IIF) and immunoprecipitation assays. After the identification of the main autoantigenic proteins, PM/Scl-100 and PM/Scl-75 (8,9), both of these proteins were shown to be homologous to bacterial exoribonuclease proteins (RNase D and RNase PH, respectively) (10).

In recent years, the PM/Scl complex was shown to be the human equivalent of the yeast exosome, a complex consisting of at least ten proteins, all displaying characteristics of exoribonucleases, which has been shown to be involved in the degradation and processing of many different RNA species (106). Besides PM/Scl-75 and PM/Scl-100 a significant number of other subunits of the human exosome have been identified, based on their homology with the yeast counterparts or by co-purification with known human exosome components (14,17).

Although PM/Scl-100 and PM/Scl-75 contain the main autoantigenic epitopes of the human exosome, also some of the other subunits have been shown to be targeted by autoanti-

bodies. Autoantibodies against the hRrp4p protein are present in 54% of the sera that are reactive with PM/Scl-100, a prevalence similar to that of anti-PM/Scl-75 autoantibodies (79). The exosome components hRrp40p, hRrp41p, hRrp42p, hRrp46p and hCsl4p all are significantly less autoantigenic, although autoantibodies recognizing these proteins have been described (79).

In general, autoantibodies against the PM/Scl complex are found in approximately 24% of patients with the polymyositis/scleroderma overlap syndrome (68), and in only 2% of patients suffering from scleroderma alone (68) and 6% of patients with myositis (polymyositis or dermatomyositis) alone (77). Conversely, between 43% and 88% of the patients positive for anti-PM/Scl antibodies are indeed diagnosed with a myositis/scleroderma overlap syndrome (68,73).

Only limited information is available on the exact epitopes on the PM/Scl-75 and PM/Scl-100 proteins that are recognized by the patient sera. The PM/Scl-100 protein appears to contain two autoantigenic epitopes (aa 229-244 and aa 775-795), both enriched in positively and negatively charged amino acid residues (9). The exact autoantigenic epitope on the PM/Scl-75 protein is not known, although most likely the highly charged C-terminal part of the protein is recognized by a subset of the autoantibodies (8). Recently, we have shown that the major isoform of the PM/Scl-75 polypeptide is 84 amino acids longer than was believed before, by characterizing cDNAs encoding an additional N-terminal part that is missing in the originally described sequence (8), but which is present in the reported PM/Scl-75 sequence of *Mus musculus*. This domain was found to be required for the incorporation of PM/Scl-75 in the core of the exosome complex (129). This exosome core consists of a ring of 6 proteins, which are all homologous to RNase PH (106). A schematic illustration of the difference between the original (PM/Scl-75a) and the longer (PM/Scl-75c) form of the protein is depicted in Figure 1. In this study, we compared

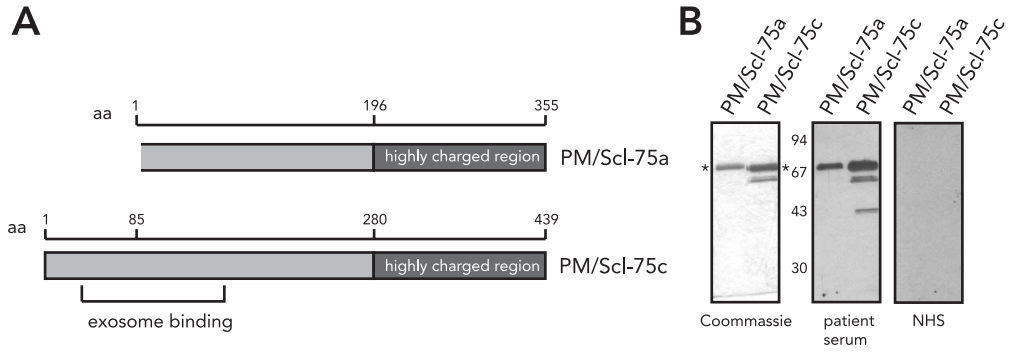


**Figure 1. The difference between PM/Scl-75a and PM/Scl-75c.**
A schematic overview of the difference between the originally published PM/Scl-75 polypeptide (8) and the full-length polypeptide that has been described recently (129) is shown in panel A. The full-length PM/Scl-75 protein has 84 additional amino acids at its N-terminus that are required for the association of this protein with the exosome complex. The highly charged C-terminal region of the protein, suggested to be important for its autoantigenicity, is also indicated. The numbers refer to amino acid (aa) positions in the respective polypeptides. A third form of the protein, called PM/Scl-75b (129), which contains only part of the additional N-terminal sequence of PM/Scl-75c, is not included in this study. Panel B shows a Coommassie stained gel with His-tagged recombinant PM/Scl-75a and PM/Scl-75c and blots containing the same proteins, stained with an anti-PM/Scl-75 positive patient serum and a normal human serum (NHS). Both full-length recombinant proteins are indicated with an asterix.

the autoantigenicity of these two forms of PM/Scl-75 in parallel with that of PM/Scl-100 in patients with the PM/Scl overlap syndrome and various other diseases in a recombinant protein ELISA.

## Results

To assess the reactivity of patient sera with PM/Scl-75a (8), PM/Scl-75c (129) and PM/Scl-100 (9), His-tagged recombinant proteins were expressed in *E. coli* and subsequently purified. Figure 1B shows a Coommassie stained gel of the recombinant PM/Scl-75 proteins. A reference anti-PM/Scl-75 patient serum was reactive with both recombinant proteins on western blot (Figure 1B). Note that the PM/Scl-75a protein runs at a similar size compared to PM/Scl-75c due to the fusion of a vector-encoded sequence to the open reading frame of the former. These proteins were used as a substrate in an ELISA to detect antibodies reactive with these proteins present in the sera of 45 patients with PM, 43 patients with DM, 51 patients with Scl and 36 patients with the PM/Scl overlap syndrome. In addition, the sera of 30 patients suffering from malignant melanoma were tested in this assay, because hRrp46p, another subunit of the PM/Scl complex, was recently shown to be autoantigenic in patients with various types of cancer, including melanoma (80). Table 1 summarizes the reactivity of patient sera with each of the proteins. In total, 8% (16/205) of the sera was reactive with PM/Scl-75c, whereas only 2% (4/205) was reactive with PM/Scl-75a, indicating that the N-terminal part of PM/Scl-75 indeed plays an important role in the autoantigenicity of the protein. The prevalence of the anti-PM/Scl-75c antibodies was the highest in patients suffering from the PM/Scl overlap syndrome (28%, 10/36), but they were also detected in some sera of patients with DM (2%, 1/43) or Scl (10%, 5/51). No anti-PM/Scl-75c reactivity was found in patients with PM or melanoma. Notably, anti-PM/Scl-100 autoantibodies were detected in only 25% (9/36) of the patients with PM/Scl and also in 2% of the patients with either PM (1/45), DM (1/43) or Scl (1/51) and in 7% (2/30) of the patients with melanoma.

Figure 2A shows the reactivity of patient sera with the antigens, compared to a number of control sera (the antibody ratio is calculated by dividing the observed reactivity by the cut-off value). It is evident that patients with the PM/Scl overlap syndrome that are positive for PM/Scl-75c generally have higher titers of these antibodies than patients with scleroderma.

Several other techniques (including western blotting, line immunoassay (LIA) and immunoprecipitation) were used to analyze the reactivity of patient sera with the recombinant PM/Scl-75

**Table 1. Reactivity of patient sera with PM/Scl-75a, PM/Scl-75c and PM/Scl-100.**

| | sera tested | PM/Scl-75a (%) | PM/Scl-75c (%) | PM/Scl-100 (%) |
|---|---|---|---|---|
| polymyositis (PM) | 45 | 0 (0) | 0 (0) | 1 (2) |
| dermatomyositis (DM) | 43 | 0 (0) | 1 (2) | 1 (2) |
| scleroderma (Scl) | 51 | 0 (0) | 5 (10) | 1 (2) |
| PM/Scl overlap | 36 | 4 (11) | 10 (28) | 9 (25) |
| melanoma | 30 | 0 (0) | 0 (0) | 2 (7) |
| total | 205 | 4 (2) | 16 (8) | 14 (7) |

proteins. In part, the ELISA data were confirmed by these experiments. However, some sera that were not reactive with PM/Scl-75a in ELISA displayed a (weak) reactivity with the PM/Scl-75a protein in these assays. This is most likely due to the presence of relatively low titers of autoantibodies directed to the C-terminal epitopes of the protein, which remain undetected in ELISA. On the other hand, for some sera no reactivity with PM/Scl-75 was detected at all, suggesting that the recognition of at least one important epitope of the protein is highly conformation-dependent, which is in agreement with previous observations (15). In conclusion, ELISA appears to be the method of choice to compare the reactivity of sera with the two isoforms of PM/Scl-75.

The co-occurrence of anti-PM/Scl-75 and anti-PM/Scl-100 antibodies in positive patient sera is depicted in Figure 2B. In agreement with previous observations, all sera that are reactive with PM/Scl-75a are also reactive with PM/Scl-100 (79). No sera were found that are reactive with PM/Scl-75a but not with PM/Scl-75c, which is not surprising because the complete amino acid sequence of PM/Scl-75a is also present in PM/Scl-75c. Importantly, both in the total group of reactive sera and the subset of PM/Scl overlap sera, sera that show reactivity with only PM/Scl-100 (4/20 of all reactive sera) or

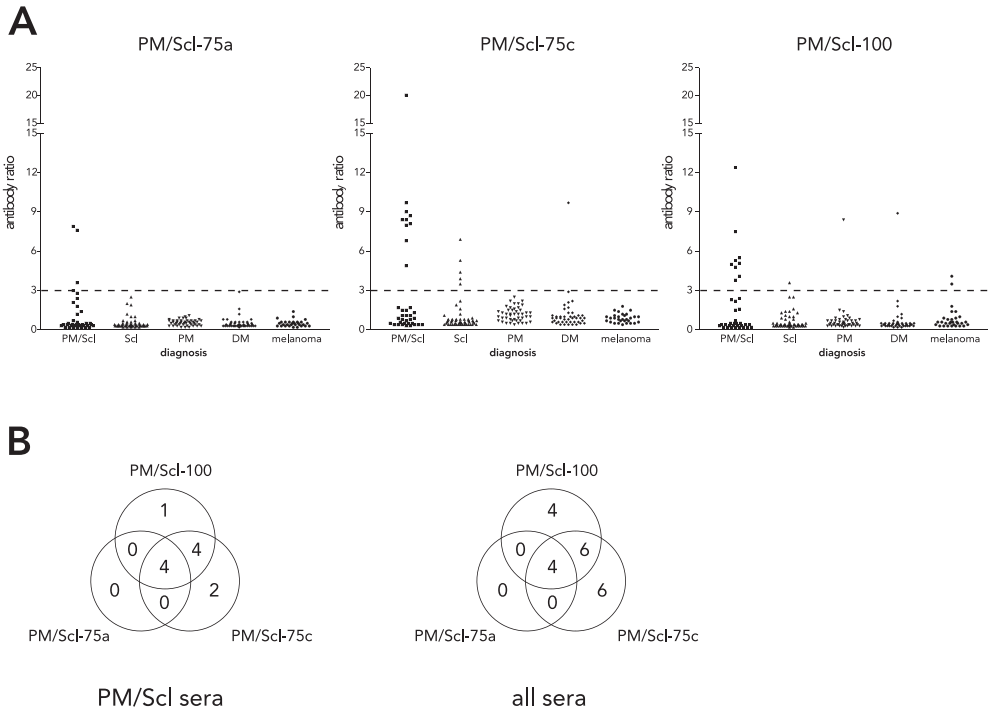


**Figure 2. Reactivity of patient sera with recombinant PM/Scl-75a, PM/Scl-75c and PM/Scl-100.**
Panel A shows the reactivity of patient sera from different patients with the recombinant proteins relative to the reactivity of normal human serum (the antibody ratio indicates the titer of the antibodies relative to the control sera). The cutoff used for positivity (a ratio of >3) is indicated by the dotted line. In panel B, the co-occurrence of autoantibodies that recognize the recombinant proteins is illustrated separately for all the sera analyzed and for the PM/Scl overlap sera.

only PM/Scl-75c (6/20) were found. Only half (50%, 10/20) of the sera reactive with PM/Scl-75 or PM/Scl-100 contains antibodies directed to both proteins, although this number is higher in the patients with the PM/Scl overlap syndrome (73%, 8/11). Combining the results obtained for PM/Scl-75c and PM/Scl-100 shows that 31% (11/36) of the PM/Scl patients are positive for either PM/Scl-75c or PM/Scl-100.

## Discussion

The aim of this study was to compare the autoantigenicity of the full-length PM/Scl-75 protein that we have described recently (129) with that of the originally defined PM/Scl-75 polypeptide described by Alderuccio *et al.* (8) and the PM/Scl-100 protein (9). The results showed that the presence of the newly identified N-terminus of PM/Scl-75 dramatically increases the frequency by which it is recognized by patient sera in ELISA. Moreover, our results suggest that autoantibodies directed to PM/Scl-75 might be more prevalent in patients with the PM/Scl overlap syndrome and related diseases than anti-PM/Scl-100 autoantibodies, which were considered to be most frequently present up to now.

In the past, the presence of these antibodies in serum was generally monitored by IIF with HEp-2 cells, immunodiffussion assays with calf thymus extract and/or by immunoblotting using extractable nuclear antigens. All these techniques allow the detection of a wide variety of autoantibodies present in patient serum. The detection of anti-PM/Scl antibodies by immunoblotting, however, is difficult, because the reactivity of the antibodies with particularly PM/Scl-75 in cell extracts is notoriously weak on immunoblots (74), which may be due to the importance of conformational epitopes. In recent years especially ELISA using a recombinant PM/Scl-100 protein has become more common to detect anti-PM/Scl reactivity, because it can easily be applied in an automated setting. In ELISA conformational epitopes in recombinant proteins are more readily formed than in other assays, allowing more sensitive detection of anti-PM/Scl-75 autoantibodies. On the other hand, the use of a stringent cut-off value, as is often required to obtain sufficient specificity, makes it difficult to detect weak reactivity with an antigen, which in the present study is particularly true for PM/Scl-75a.

Since anti-PM/Scl-75 reactivity was previously only detected in patient sera that also contained anti-PM/Scl-100 autoantibodies (74,77), this protein is usually not included in such assays. In addition to the limitations of the applied detection method, our data show that also the use of an incomplete recombinant PM/Scl-75 polypeptide may have led to an underestimation of the diagnostic value of the PM/Scl-75 antigen. Although more cohorts of PM/Scl, Scl, PM and DM sera have to be analyzed with the full-length PM/Scl-75 antigen, our data strongly suggest that, if only PM/Scl-100 is used in ELISA assays, a significant number of patients will test negative, because anti-PM/Scl-75 antibodies remain undetected. Some of these would test positive with PM/Scl-75a in other assays, whereas others might only be detected using PM/Scl-75c in ELISA. Taken together, the use of both recombinant proteins increases the number of patients with the PM/Scl overlap syndrome detected by ELISA. Although the prevalence of autoantibodies recognizing most other exosome subunits relatively low (79), the co-occurrence of antibodies targeting different exosome subunits in patient sera might be indicative for the overlap

syndrome. The co-occurrence of anti-PM/Scl-100 and anti-PM/Scl-75 seems to be particularly associated with the PM/Scl overlap syndrome (see Figure 2B), but whether the use of even more components of the human exosome will further increase the sensitivity of these assays remains to be investigated.

## Materials and Methods

### *Patients*

Most patient sera were obtained from the departments of Surgery, Rheumatology and Neurology from the University Medical Centre St. Radboud, Nijmegen. Patients were selected that met the established criteria for Scl, PM, DM or having malignant melanoma. Many of the patients with PM, DM or overlap syndromes of Scl with PM or DM (both referred to as PM/Scl in this study) were also included in previous studies that assessed the prevalence of anti-exosome antibodies in these patients (77,79).

### *Expression and purification of recombinant proteins*

The cDNA encoding PM/Scl-75a (acc. nr. M58460) was cloned into the pEx34 expression vector and the cDNAs encoding PM/Scl-75c (acc. nr. AJ505989) and PM/Scl-100 (acc. nr. X66113) were cloned into pET21a. Proteins were expressed in transformed *E. coli* 537 cells (PM/Scl-75a) and BL21(DE3)Star cells (Invitrogen) (PM/Scl-75c, PM/Scl-100) and purified by $Ni^{2+}$-affinity chromatography, followed by preparative SDS-PAGE.

### *Enzyme linked immunosorbent assay (ELISA)*

ELISA assays were essentially performed as described before (77). In brief, with 0.2 μg/well of each of the proteins, medium binding microtiter plates (Greiner, Germany) were coated and antibody screening was performed with 200 μl of diluted serum samples (1:100). After incubation (30 minutes, 37°C) and washing, plates were incubated (30 minutes, 37°C) with 200 μl/well of alkaline phosphatase-conjugated AffiniPure Fc-fragment specific goat anti-human IgG (Jackson Laboratories, USA). Bound antibodies were visualized with 38 mM disodium-paranitrophenyl-phosphate and 50 mM diethanolamine pH 9.8. ODs were measured at 405 nm and tests were regarded positive at >3 fold ODs compared to the calculated cut-off (based on three negative control sera with 99.5% confidence (133)).

# Chapter 8

## General Discussion

*Since the identification of the exosome complex, six years ago, a lot of progress has been made in the characterization of the exosome from a variety of organisms. Nevertheless, still many questions remain unanswered. What factors are responsible for the regulation of the exosome? How and where is the complex assembled and are there differences in the composition of the nuclear and cytoplasmic exosome? Why are some proteins of the exosome autoantigenic in patients with a myositis/scleroderma overlap syndrome? In this chapter, some of the most prominent questions will be discussed.*

## Structure of the exosome

The composition of both the yeast (21) and human (Chapter 3) (106) exosome has been largely identified. Both were found to contain six proteins homologous to RNase PH, which are likely to assemble into a hexameric ring. Attached to the outer surface of this ring are three proteins, each containing an S1 RNA binding domain. This exosome core structure is very similar to that of the bacterial protein polynucleotide phosphorylase (PNPase) (21,106) (Chapters 2 and 3). Very recently, also the exosome of *Drosophila melanogaster* was purified (25). Remarkably, only 4 RNase PH-like proteins were found in this complex. Since the structure of the exosome core (and the complex formed by the related protein PNPase) seems to be highly conserved throughout evolution (Chapter 4), this suggests that either two RNase PH-like proteins of the *D. melanogaster* exosome remain to be identified, or that some of the subunits are present in the complex in multiple copies. The relationship of a number of other proteins with the exosome remains unclear. The human homologue of the yeast exosome subunit Rrp44p/Dis3p, hDis3p, does not appear to be a component of the human exosome (17,106), even though this protein has been found in association with the *D. melanogaster* exosome (25). Similar to the human situation, evidence suggests that the Rrp44p/Dis3p homologue of *Trypanosoma brucei* also functions independent of the exosome (23). Homologues of the yeast subunit Rrp6, on the contrary, have been found in all exosome complexes studied to date (11,17,23,25). However, the exact nature of the interaction of this protein with the core of the exosome complex is still not understood. In yeast, the protein has been suggested to be positioned on top of the ring structure (23). This interaction might require additional proteins, since no direct protein-protein interactions between exosome subunits and Rrp6p or any of its homologues have been found so far. In the case of the human exosome, which might exist as a dimeric core complex, both rings could be involved in the interaction of one or more copies of the Rrp6p homologue PM/Scl-100 with the core of the exosome complex (see Figure 1)(106).

Quite a number of other proteins have been implicated in exosome function (Figure 1 and summarized in Chapter 1). Although preliminary studies have indicated that some of these proteins are able to associate transiently with the exosome, the exact nature of these interactions remains to be identified.

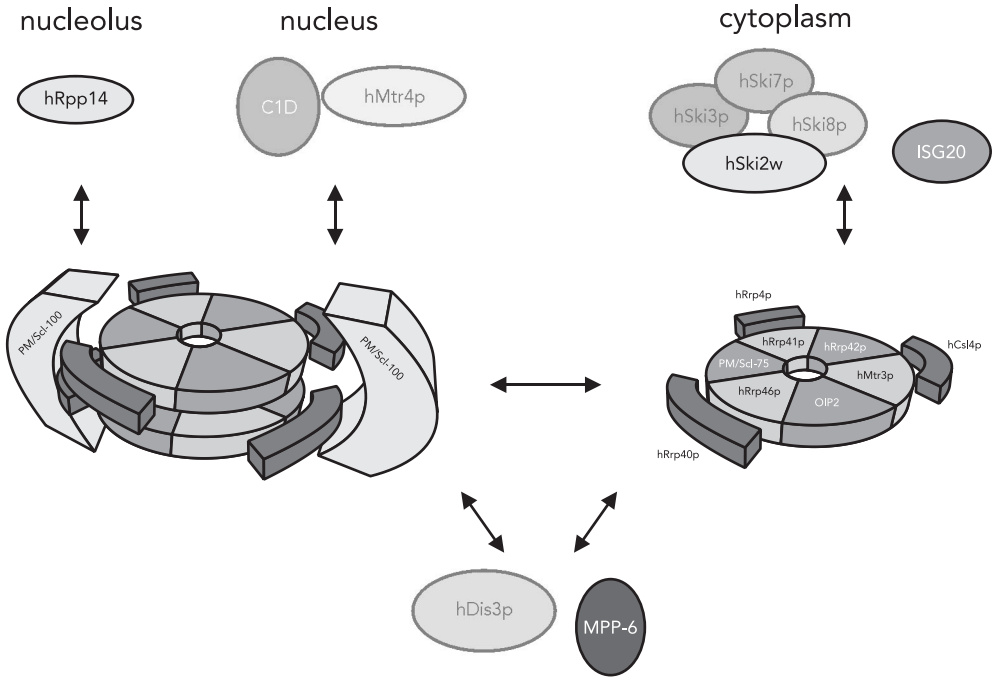


**Figure 1. Model of the human exosome and the interacting proteins.**
A schematic model of the human exosome, based on the protein-protein interactions and proteins known to interact transiently with the complex in either yeast or human. In the cytoplasm, a single exosome ring can interact with a number of regulatory proteins, whereas the nuclear exosome consists two rings. This dimerisation might be mediated by the binding of the nucleus specific exosome subunit PM/Scl-100.

## Assembly of the exosome

Only few data are available on the mode and subcellular localization of exosome assembly. Because the exosome is present in both cytoplasm and nucleus, the assembly most occurs in the cytoplasm, followed by translocation of a subset of the assembled complexes to the nucleoplasm. One of the core components, hCsl4p, has been shown to require both of its interacting partners (hRrp42p and hMtr3p) to be incorporated into the complex and to be able to enter the nucleolus (Chapter 2 and 3) (88,106). In three of the exosome subunits (hRrp41p, PM/Scl-75 and PM/Scl-100) a putative nuclear localization signal (NLS) can be identified (Chapter 5). The NLS sequences in PM/Scl-75 and hRrp41p appear to be conserved in *C. elegans* and in addition the putative NLS of hRrp41p is located in a extended loop when this protein is modelled according to the crystal structure of an RNase PH domain from *S. antibioticus* PNPase. Although the NLS present in PM/Scl-75 was able to target a reporter protein to the nucleus, this element did not appear to be essential for exosome association and nuclear localization of the protein. Still, the element might contribute to nuclear translocation of the complex *in vivo*. However, it is required for the nucleolar localization of PM/Scl-75 (129) (Chapter 5). This suggests that PM/Scl-75 is transported to the nucleus as part of an already (partially) assembled exosome complex.

Combined with the observation that PM/Scl-100 is only associated with the nuclear and not with the cytoplasmic exosome (11), these data support a model for the assembly and routing of the exosome as shown in Figure 2. Following the assembly of the ring structure in the cytoplasm, the exosome can be transported to the nucleus, possibly via the NLS present in hRrp41p. In the nucleus, the 'NLS' of PM/Scl-75 directs the complex to the nucleolus. The nucleus-specific exosome protein PM/Scl-100 is imported independently into the nucleus, where it associates with the core of the exosome complex. During these processes, fractions of the exosome are retained in the cytoplasm and nucleoplasm. The molecular mechanisms underlying the retention in cytoplasm and nucleoplasm are unknown.

## Regulation of exosome function

The exosome plays a role in a variety of processes including the removal of incorrectly processed RNA molecules and the maintenance of correct levels of mRNA, rRNA and a number of small RNA species. Despite the identification of several proteins, including RNA helicases and GTPase-like proteins, that might perform a regulatory role when associated with the exo-

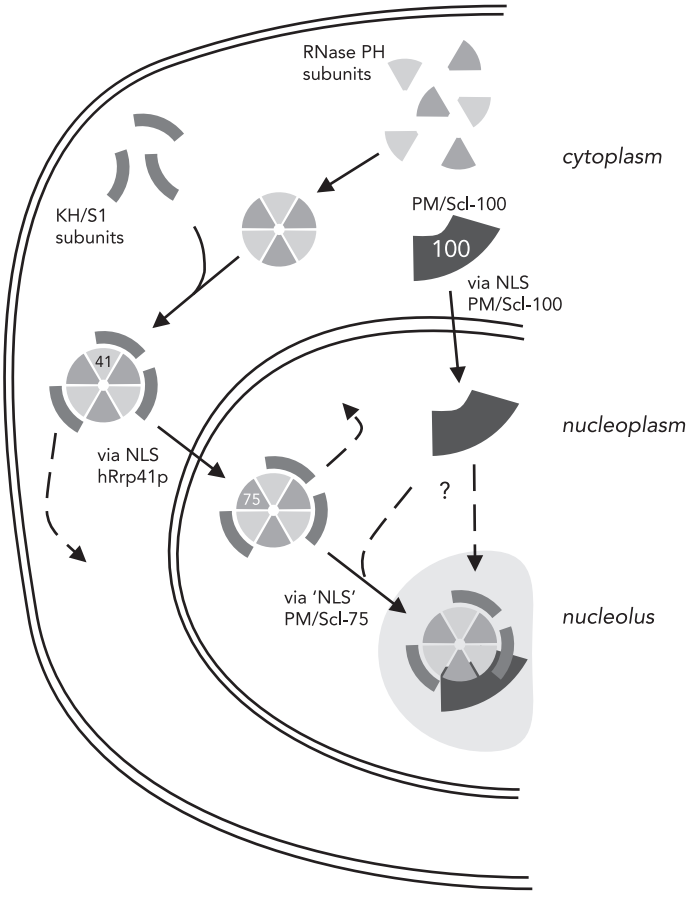


**Figure 2. Possible routing of the human exosome.**
Model for the assembly and routing of the human exosome. The exosome core most likely is assembled in the cytoplasm and a subset of the complexes is subsequently transported to the nucleoplasm. In the nucleus, the exosome core associates with PM/Scl-100 and accumulates in the nucleolus.

some (Chapter 1), the mechanisms of substrate specificity and up- an down-regulation of exosome levels are still a mystery. An important factor in determining a particular exosome function is the subcellular localization of the complex. It has been shown that distinct proteins associate with the core of the complex in the cytoplasm and nucleus. In the cytoplasm, the exosome core can interact with the Ski-complex in yeast and several AU binding proteins in human cells, all known to be involved in the degradation of mRNA (17,29). Similarly, the nuclear exosome is able to associate with a number of proteins known to be involved in rRNA processing, RNA transcription, pre-mRNA quality control and other processes involving nuclear RNA (25,27,46). Since the exosome core itself does not appear to have any sequence specificity, recognition of the substrate RNA molecules most likely involves these associated proteins, after which the RNA is handed-over to the core complex for degradation or processing.

## Origin of the exosome

The theory of the 'RNA world', in which RNA molecules were the first biomolecules capable of catalyzing enzymatic reactions and self-replication, suggests that early proteins would be able interact somehow with RNA. It is therefore not surprising that the exosome, as one of the key players in the cellular regulation of RNA metabolism, belongs to a family of structurally highly conserved complexes present in the vast majority of all known organisms (Chapter 4). The strong conservation of these enzymes in all three kingdoms of life (archaebacteria, eubacteria and eukaryotes) not only suggests that this RNA degrading machinery appeared very early in evolution, but it also stresses the importance of maintaining correct RNA levels for any organism. Although other enzymes performing similar functions are present in many organisms, none of these are as widely distributed as the exosome. The observation that many organisms do not seem to require such additional RNA degrading proteins, underscores the efficiency and universal functionality of the exosome/PNPase.

## Autoantigenicity of the exosome

One of the major molecular characteristics of autoimmune diseases is that the patients produce autoantibodies recognizing certain self-proteins. In many cases the targeted autoantigen is strongly correlated with the disease and determination of the specificity of the autoantibodies can therefore facilitate the diagnosis. In rheumatoid arthritis, for example, the majority of the patients produce autoantibodies recognizing epitopes containing the non-standard amino acid citrulline, whereas autoantibodies with this specificity are not found in healthy individuals or in patients with other autoimmune diseases (134). Although it is hypothesized that the generation of autoantibodies could be linked to cell death following cell damage or inflammation (125), the remarkable relation between a disease and the specificity of the antibodies associated with that disease is still not understood. Many autoantigens have been shown to be modified or cleaved during either apoptosis or necrosis, thereby possibly triggering the immune system. But how a modified autoantigen is capable of inducing a specific autoimmunedisease remains a unclear, although the genetic background (especially the HLA genes) of an individual might be of great importance.

Autoantibodies recognizing the exosome, and in particular the PM/Scl-75 and PM/Scl-100 subunits, are found in approximately 25% of all patients suffering from the overlap syndrome of two autoimmune diseases, myositis and scleroderma (Chapter 7) and the presence of these antibodies often correlates with a HLA-DR3 genotype (78). Since the exosome most likely is present inside all cells, its subunits can only be exposed to the immune system following cell damage. Interestingely, at least one of the exosome components, PM/Scl-75, appears to be modified during apoptosis (Chapter 6). Although this could provide a clue towards understanding the development of the disease and the occurrence of anti-exosome autoantibodies, many additional studies will be required to understand the phenomenon of autoimmunity.

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## Summary

The human exosome is a multi-protein complex present in all eukaryotic cells, which is involved in the processing and degradation of a large variety of RNA molecules. It plays a central role in the regulation and maintenance of correct levels of messenger RNA, ribosomal RNA and other RNA species.

In patients suffering from an overlap syndrome of the autoimmune diseases polymyositis (PM) and scleroderma (Scl), autoantibodies are found that are reactive with protein subunits of this complex. These antibodies are referred to as anti-PM/Scl antibodies.

The goal of this study was to characterize in more detail the composition and structure of the human exosome and to gain more insight in the reason why this complex is targeted by autoantibodies in patients with the PM/Scl overlap syndrome.

**Chapter 1** gives an overview of our current knowledge on the composition, structure and the various functions of the exosome. Although the exosome of yeast has been studied most extensively, more information has recently become available regarding the exosome in other organisms, including men.

The interactions between three of the components of the human exosome, hCsl4p, hRrp42p and hRrp46p, are described in detail in **Chapter 2**. The hCsl4p protein was shown to interact directly with both other proteins *in vitro* and using deletion mutants of the protein the regions responsible for this interaction were determined. A systematic analysis of interactions between human exosome subunits is presented in **Chapter 3**, together with an interesting homology between the exosome and the bacterial protein polyribonucleotide phosphorylase (PNPase). The combination of these data allowed the construction of a model for the exosome complex, the main feature of which is a ring structure composed of the six exosome subunits that contain an RNase PH domain.

A human homologue of the bacterial PNPase protein is described in **Chapter 4**. This protein is localized in the mitochondria and is an active ribonuclease, both *in vivo* and *in vitro*. Furthermore, the evolutionary relationships between the exosome complex and both prokaryotic and eukaryotic PNPases are discussed.

**Chapter 5** deals specifically with one of the exosome subunits, PM/Scl-75, which is a significantly longer protein than was believed until recently. The additional N-terminal sequence was shown to be important for the nucleolar localization of this protein as well as for the interaction between PM/Scl-75 and the rest of the exosome complex. PM/Scl-75 is also a substrate for caspase-mediated cleavage in apoptotic cells (**Chapter 6**), suggesting a role for the protein in the generation of patient autoantibodies that recognize the exosome complex. PM/Scl-75 is most likely cleaved in its C-terminal region, and the cleavage can probably occur when the protein is associated with the complex.

In **Chapter 7** it is shown that autoantibodies from patients with the PM/Scl overlap syndrome are mainly targeted to the longer form of the PM/Scl-75 protein, whereas until now another exosome subunit, PM/Scl-100, was believed to be the main autoantigen of the complex.

The results obtained in this project and the questions that remain to be answered are discussed in **Chapter 8**.

## Samenvatting

Het menselijke exosoom is een complex bestaande uit een tiental eiwitten dat aanwezig is in alle eukaryotische cellen. Het is betrokken bij het "op maat snijden" en het volledig afbreken van allerlei RNA moleculen en speelt een belangrijke rol bij het in stand houden van de correcte hoeveelheden messenger RNA, ribosomaal RNA en andere soorten RNA.

Bij patiënten die lijden aan het overlapsyndroom van de auto-immuunziekten polymyositis (PM) and sclerodermie (Scl) worden autoantilichamen gevonden die gericht zijn tegen eiwitten van dit complex. Daarom worden deze antilichamen ook wel anti-PM/Scl antilichamen genoemd. Het doel van het onderzoek was om de samenstelling en structuur van het humane exosoom te bepalen en om meer inzicht te krijgen in de vraag waarom er in patiënten met het PM/Scl overlapsyndroom autoantilichamen gericht tegen dit complex geproduceerd worden.

**Hoofdstuk 1** beschrijft de huidige kennis op het gebied van de samenstelling, structuur en de verschillende functies van het exosoom. Hoewel het exosoom vooral in gist bestudeerd is, werd er recentelijk ook steeds meer bekend over de structuur en functie van het exosoom in andere organismen, waaronder dat van de mens.

De interacties tussen drie van de eiwitten van het humane exosoom, hCsl4p, hRrp42p en hRrp46p, worden beschreven in **Hoofdstuk 2**. Het hCsl4p eiwit gaat *in vitro* een directe interactie aan met de twee andere eiwitten, en met behulp van deletiemutanten van het eiwit is bepaald welke gebieden van het eiwit voor deze interactie verantwoordelijk zijn. Een systematische analyse van alle interacties tussen exosoom eiwitten is beschreven in **Hoofdstuk 3**. Ook wordt in dit hoofdstuk een verrassende overeenkomst tussen het exosoom en het bacteriële eiwit polyribonucleotide phosphorylase (PNPase) beschreven. Deze laatste ontdekking maakte het mogelijk een model voor de structuur van het exosoom te ontwerpen, waarbij de zes exosoom eiwitten met een RNase PH domein een ringstructuur vormen die de kern van het complex vormt.

Een humane homoloog van het bacteriële PNPase eiwit wordt beschreven in **Hoofdstuk 4**. Dit eiwit is aanwezig in de mitochondria en is een actief exoribonuclease, zowel *in vitro* als *in vivo*. In dit hoofdstuk wordt ook de evolutionaire relatie tussen het exosoom en het PNPase van zowel prokaryoten als eukaryoten behandeld.

**Hoofdstuk 5** concentreert zich op één van de exosoomeiwitten, PM/Scl-75, waarvan wordt aangetoond dat het een aanzienlijk groter eiwit is dan tot nu toe werd aangenomen. De extra sequentie aan de N-terminus van het eiwit blijkt belangrijk te zijn voor zowel de accumulatie van het eiwit in de nucleoli als voor de interactie van PM/Scl-75 met de rest van het complex. PM/Scl-75 is ook een substraat voor klieving door caspase enzymen in apoptotische cellen (**Hoofdstuk 6**), wat suggereert dat het eiwit betrokken zou kunnen zijn bij het ontstaan van autoantilichamen gericht tegen het exosoom. Het eiwit wordt waarschijnlijk in de C-terminale regio gekliefd, vermoedelijk terwijl het onderdeel uitmaakt van het exosoom complex.

In **Hoofdstuk 7** wordt beschreven dat de autoantilichamen in patiënten met het PM/Scl overlap syndroom met name gericht zijn tegen de langere vorm van PM/Scl-75. Tot nu toe werd aangenomen dat een ander exosoom eiwit, PM/Scl-100, het belangrijkste autoantigen van het complex was.

Tot slot worden de resultaten uit dit proefschrift en de nog onbeantwoorde vragen bediscussieerd in **Hoofdstuk 8**.

## Eenvoudige samenvatting

Het was in 2003 precies 50 jaar geleden dat James Watson en Francis Crick hun inmiddels wereldberoemde helixstructuur voor het erfelijke materiaal DNA publiceerden. Tot dat moment was grotendeels onduidelijk hoe erfelijke informatie in het DNA wordt opgeslagen en hoe erfelijke informatie van ouders op nakomelingen wordt overgedragen. Sinds de ontdekking van de DNA structuur is veel vooruitgang geboekt bij de beantwoording van deze belangrijke vragen. Zo weten we nu dat de informatie die in het DNA opgeslagen is (de 'genen') gekopieerd wordt in een vergelijkbaar molecuul, genaamd RNA, dat op zijn beurt weer vertaald wordt in een eiwit. Het zijn de eiwitten die de meeste belangrijke functies in onze cellen uitvoeren.

In dit proefschrift staan enkele van deze eiwitten centraal. De door mij bestudeerde eiwitten (exosoom eiwitten) hebben als belangrijkste functie het RNA af te breken wanneer dat nodig is, en kunnen op die manier de overdracht van informatie van DNA naar eiwit beïnvloeden.

De exosoom eiwitten zijn ook in medisch opzicht interessant. In patiënten die lijden aan de ziekten polymyositis en sclerodermie is het immuunsysteem dikwijls zodanig ontregeld dat het lichaam antistoffen tegen deze exosoom eiwitten maakt. Normaal gesproken is het immuunsysteem alleen gericht tegen lichaamsvreemde stoffen (bijvoorbeeld afkomstig van infecties door bacteriën of virussen) en zal het geen lichaamseigen eiwitten aangrijpen. Bij auto-immuunziekten gebeurt dit wel, en valt het immuunsysteem onze eigen eiwitten en cellen aan. Welke eiwitten, cellen of weefsels worden aangevallen hangt af van de auto-immuunziekte waar de patiënt aan lijdt. Bij myositis worden de spieren aangetast; bij sclerodermie is dat de huid en het onderliggende bindweefsel. Bij het polymyositis/sclerodermie overlap syndroom uit zich dat, naast de schade aan spier- en bindweefsel, blijkbaar in de vorming van antistoffen die gericht zijn tegen het exosoom.

Van de eiwitten die in dit proefschrift bestudeerd zijn was aan het begin van het project al bekend dat ze onderdeel uitmaakten van een groter complex van tenminste tien eiwitten dat het exosoom werd genoemd (**Hoofdstuk 1**). Het was echter niet bekend hoe dit complex of de verschillende onderdelen ervan er precies uitzagen en waarom sommige patiënten er antistoffen tegen maken.

In de **Hoofdstukken 2 en 3** hebben we bepaald wat de vermoedelijke vorm van dit complex zou kunnen zijn. Het lijkt er op dat het exosoom een ringvorm aanneemt, die uit zes eiwitten bestaat, met nog drie eiwitten die aan de buitenkant op deze ring geplakt zitten. Voor één van de tien eiwitten kon nog geen plekje in dit model gevonden worden. De hier voorgestelde ringvorm lijkt heel erg op de structuur van een ander eiwit, genaamd PNPase, waarvan oorspronkelijk werd gedacht dat het alleen in bacteriën en planten voorkwam. Dit eiwit blijkt ook in de mens aanwezig te zijn, zoals beschreven is in de **Hoofdstukken 3 en 4**.

De laatste drie hoofdstukken gaan over een van de meest interessante componenten van het exosoom, het eiwit PM/Scl-75. Het is een van de eiwitten waartegen antistoffen voorkomen in de al eerder genoemde auto-immuun patiënten. **Hoofdstuk 5** beschrijft de verschillende vormen die van dit eiwit gevonden zijn en toont ook aan dat het eiwit in werkelijkheid groter is dan tot nu toe werd aangenomen. Dat deze langere vorm van PM/Scl-75 ook inderdaad een rol speelt bij de auto-immuniteit van patiënten

staat beschreven in **Hoofdstuk 7**. Bij patiënten met zowel myositis als sclerodermie blijken de aanwezige autoantistoffen voornamelijk gericht te zijn tegen deze langere vorm van PM/Scl-75, een aanwijzing is dat dit eiwit misschien een rol speelt bij het ontstaan van de autoantistoffen. Een mogelijk mechanisme daarvoor wordt in **Hoofdstuk 6** gesuggereerd. We beschrijven daar dat een deel van het eiwit PM/Scl-75 wordt afgeknipt tijdens het doodgaan van cellen (een proces dat veel voorkomt bij de ontstekingen in auto-immuunziekten). Het kan zijn dat het verkorte eiwit door het immuunsysteem als "vreemd" wordt herkend waardoor er antistoffen tegen gemaakt worden. Of dit inderdaad het mechanisme is dat leidt tot de vorming van deze antistoffen zal nog meer onderzoek vergen. Wel kan het bepalen of autoantistoffen tegen PM/Scl-75 aanwezig zijn in patiënten in de toekomst bijdragen aan het diagnosticeren van patiënten met het PM/Scl overlap syndroom.

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- **Pruijn, G.J.M., Schilders, G. and Raijmakers, R.** The exosome and PNPase, an ancient family of RNA shredders. *Recent Research Developments in Molecular Biology*: in press
- **Raijmakers, R., Peeters, L., Vree Egberts, W., van Venrooij, W.J. and Pruijn, G.J.M.** The origin of mitochondrial polynucleotide phosphorylase. *submitted for publication*
- **Raijmakers, R., Vogelzangs, J., Croxford, J.L., Wesseling, P., Pruijn, G.J.M and van Venrooij, W.J.** Citrullination of central nervous system proteins during the development of EAE. *submitted for publication*

## Curriculum Vitae

Reinout Raijmakers is geboren op 30 januari 1976 te Oss. In 1994 behaalde hij zijn Atheneum-B diploma aan het Titus Brandsmalyceum te Oss. In september van dat jaar begon hij zijn studie Scheikunde aan de Katholieke Universiteit Nijmegen (KUN). Tijdens deze studie heeft hij zijn bijvakstage gedaan bij de afdeling Bio-Organische Chemie in Utrecht (dr. C.J.M. Stroop, prof. dr. J.P. Kamerling en prof. dr. J.F.G. Vliegenthart, Universiteit Utrecht) en zijn hoofdvakstage bij de afdeling Biochemie NWI (mw. dr. S.A. Rutjes, dr. G.J.M Pruijn en prof. dr. W.J. van Venrooij, KUN). Als onderdeel van deze hoofdvakstage heeft hij enkele maanden onderzoek gedaan op het Vienna Biocenter (dr. G. Fabini, dr. G. Steiner, Universiteit Wenen, Oostenrijk). Het doctoraalexamen Scheikunde heeft hij gehaald in april 1999.

Van mei 1999 tot mei 2003 was hij via een NWO-subsidie werkzaam als Assistent in Opleiding (AiO) bij de afdeling Biochemie van de Faculteit Natuurwetenschappen, Wiskunde en Informatica van de KUN, onder begeleiding van dr. G.J.M Pruijn en prof. dr. W.J. van Venrooij. In die periode werd het onderzoek, beschreven in dit proefschrift, uitgevoerd. Sinds mei 2003 werkt hij op ditzelfde lab als post-doc aan een project gericht op de rol van eiwitmodificaties in het ontstaan van type I diabetes en multiple sclerose.

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En nu, beste lezer, bent u aan het einde beland van dit proefschrift. Het doet mij goed dat u, geboeid door de wetenschappelijke verhandelingen beschreven in de voorgaande hoofdstukken, dit boekwerk tot het einde heeft weten uit te lezen. Daar wil ik u dan ook van harte voor bedanken, net als de vele mensen die hebben bijgedragen aan het tot stand komen van dit proefschrift.

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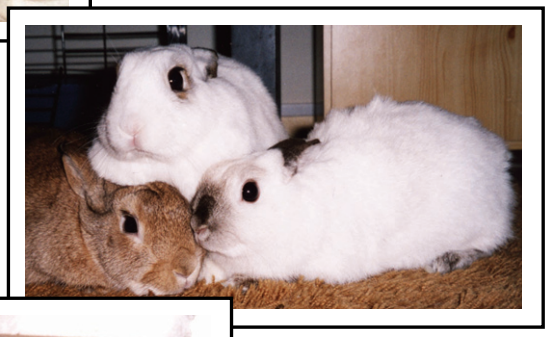
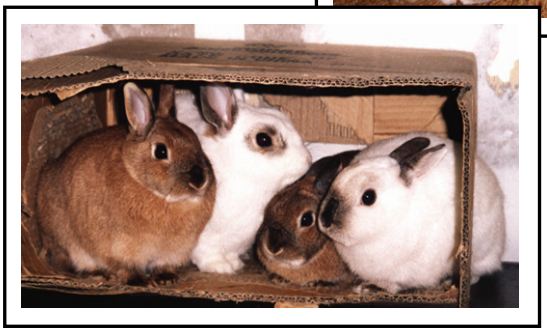
**Reinout**





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